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PANCREATIC CANCER

A clinical and experimental study of
pathogenetic and prognostic factors
with special emphasis on
hormonal influences



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by

Åke Andrén-Sandberg

Lund 1983

This is dedicated to the ones I love



The present thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

- I. Andrén-Sandberg Å, Ihse I.
Factors influencing survival after total pancreatectomy in patients with pancreatic cancer.
Ann Surg, in press 1983.
- II. Andrén-Sandberg Å, Dawiskiba S, Ihse I.
Sequential studies on the development of nitrosamine-induced pancreatic carcinoma in the Syrian golden hamster.
Acta Pathol Microbiol Scand Sect A, in press.
- III. Andrén-Sandberg Å, Hultberg B, Ihse I.
Changes in pancreatic digestive and lysosomal enzymes during induced pancreatic carcinogenesis.
Submitted.
- IV. Andrén-Sandberg Å, Hultberg B, Ihse I.
Influence of N-nitrosobis(2-oxypropyl)amine (BOP) on pancreatic secretion in the Syrian golden hamster.
Submitted.
- V. Andrén-Sandberg Å, Dawiskiba S, Ihse I.
Effect of bili-digestive anastomosis on experimental pancreatic carcinogenesis.
Acta Chir Scand 148:511-515, 1982.
- VI. Andrén-Sandberg Å, Ihse I.
Regulatory effects on the pancreas of intraduodenal pancreatic juice and trypsin in the Syrian golden hamster.
Scand J Gastroenterol 18:697-706, 1983.
- VII. Andrén-Sandberg Å, Dawiskiba S, Ihse I.
Studies on the effect of cerulein administration on experimental pancreatic carcinogenesis.
Scand J Gastroenterol, in press.
- VIII. Andrén-Sandberg Å, Dawiskiba S, Ihse I.
Studies on the effect of cholecystokinin, secretin and oral trypsin inhibitor administration on experimental pancreatic carcinogenesis.
Submitted.
- IX. Andrén-Sandberg Å, Borg Å, Fernö M, Dawiskiba S, Ihse I.
Studies on the fitness of the Syrian golden hamster for investigations of estrogen effects on induced pancreatic carcinogenesis.
Submitted.

- X. Andrén-Sandberg Å, Björk P, Gunnarsson PO, Ihse I.
Uptake and binding of estramustine in nitrosamine-induced
exocrine pancreatic cancer in the Syrian golden hamster.
Submitted.

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In the past research on pancreatic cancer has been hampered by a defeatist attitude to the disease as a consequence of the dismal prognosis. There are still no effective ways to make an early diagnosis or to screen large populations, and the result of any kind of therapy has been discouraging. Besides, there is a lack of basic information on etiology and pathophysiology and, until recently, no appropriate experimental model has been available.

During the last few years, however, there has been an increasing interest in problems related to pancreatic cancer, partly due to the development of an experimental model for induction of ductal pancreatic cancer. Also, the increase in incidence of the disease all over the world has made us understand that pancreatic cancer today is not only a qualitative problem but also a quantitative one. The present work is to be looked upon as an expression of a growing optimism aiming at an improved outlook for the patient with pancreatic cancer.

2. CHAPTER I: BACKGROUND

2.1. Pathology

Among all pancreatic tumors only 1 % are benign and 99 % of the malignant tumors are emanating from the exocrine pancreas (1). According to Cubilla and Fitzgerald 89 % of the cancers can be classified as ductal/ductular cancer, 1 % as acinar cell carcinoma, 1 % as connective tissue cancers, whereas the histogenesis in 9 % is uncertain (2). Pancreatic cancer is localized to the head of the gland in 61 % of the patients, to the body in 13 %, to the tail in 5 % and to more than one of these areas in the remaining 21 % (3). The mean diameter of cancers of the head of the pancreas at the time of diagnosis has been estimated to 5 cm (3, 4). The site of origin of large tumors of the ampulla-head-region is often difficult to determine but usually 75 % of the lesions at the choledocho-duodenal junction are judged to be exocrine pancreatic cancers (4) and the remaining cancers to be originating from the duodenum, the papilla of Vater or the choledochus. These latter tumors have a significantly better prognosis (5-7) and it is therefore important to look upon them as different from pancreatic cancer.

In this thesis the term pancreatic cancer only implies exocrine pancreatic cancer and not endocrine cancer nor cancer originating from the duodenum, the papilla of Vater or the distal bile duct.

2.2. Epidemiology

2.2.1. Global incidence

A source of error in epidemiological studies - of certain importance in pancreatic cancer - is the low frequency of histological verification of the diagnosis (8), and the fact that the diagnosis is sometimes made first at autopsy (8-10). Therefore, a critical attitude towards all epidemiological studies is advisable.

A collection of international data from around 1970 and age-adjusted to a world standard population shows that in USA, England, Israel and the Scandinavian countries the incidence of pancreatic cancer is very high (8), but the highest figures are found in male Hawaiians on Hawaii (15.8 per 100 000 inhabitants). The highest incidence for women is that of Spanish descent in the El Paso-district in USA (9.2 per 100 000 inhabitants). Mortality data from 34 countries collected by Aoki and Ogawa 1978 (11) clearly show an increase of pancreatic cancer during the last 30 years, both of the total number and of the age-adjusted number of cases.

However, there has been a tendency to leveling off after 1970 in countries with high incidence rates. As pancreatic cancer is more common in higher ages (8-10) the total increase is most pronounced in

an aging population, but there has also been shown an increase in a cohort study (12).

The highest proportion of pancreatic cancer in relation to the total number of all cancers is found in males of Spanish descent in New Mexico (6.1 %) and Maoris on New Zealand (5.5 %) and the highest proportion in females is found in USA: blacks in Almeida (4.2 %) and in Bay Area (3.9 %). Incidence rates are usually higher for men than for women. The male/female ratio is about 1.5, ranging from 0.4 (American Indians in New Mexico) to 3.2 (Malta) (8). In USA there has been a change in male/female ratio from 1.26 in 1935, raising gradually to a peak of 1.45 in 1955, and then again declining to a ratio of 1.25 in 1973. Obviously part of the decline during the last two decades depends on the greater, and increasing, number of women at more advanced ages (13).

As pointed out by Berg and Connelly (14) the variation of incidence rates for pancreatic cancer between countries is one of the smallest among all cancers; unfortunately this implies that epidemiological studies of this disease have been of limited value.

2.2.2 Incidence in Sweden

Sweden belongs to a group of countries with a high age-adjusted

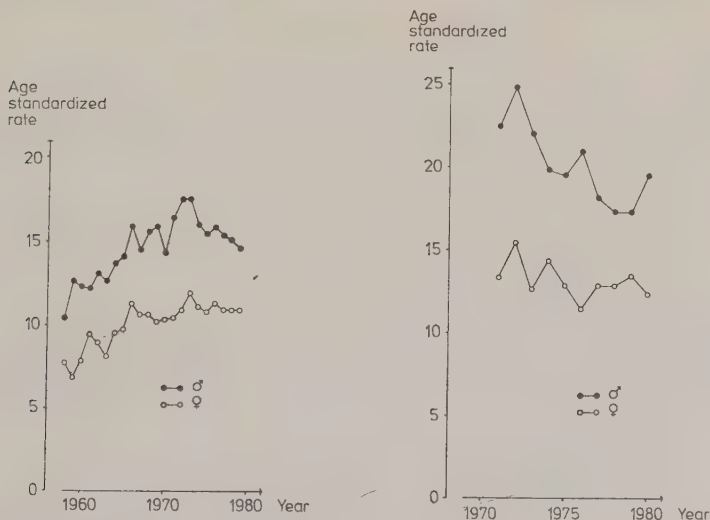


Fig 1. To the left age-standardized incidence for pancreatic cancer in Sweden 1958-1979. To the right age-standardized incidence for pancreatic cancer in Stockholm 1969-1970 (Source: The Cancer Registry, National Board of Health and Welfare, Stockholm, Sweden).

pancreatic cancer incidence: 14.6/100 000 men and 10.9/100 000 women 1979 (15), corresponding to 3.5 % of all malignant diseases in men and 3.3 % in women. The absolute annual number of pancreatic cancer diagnoses has increased continuously during the years since compulsory reporting to the Swedish Cancer Registry started. Thus, in 1958 618 cases were diagnosed and 1.157 cases in 1979 (15, 16). As the disease most of all affects the elderly (8-10) the age standardized incidence has displayed a different time trend. Contrary to the continuing increase during the 1960's the increasing rate has slowed down among women whereas a tendency to decrease can be noted among men (Fig. 1). In Stockholm, where the incidence is far above the average in Sweden, a decreasing trend is noted both for men and women during the last decade (Fig. 1).

However, even if the incidence is leveling off in Sweden the high total number of newly diagnosed cases with this fatal disease is a considerable resource problem.

2.3 Risk factors

2.3.1. Demographic risk factors

The most important single factor associated with an increased risk for pancreatic cancer is age (8-16). However, incidence data from various parts of USA have shown that the once large excess risk for urban residents has diminished over time, but the urban/rural differences are still fairly large in several European countries (8), including Sweden (9, 15, 16). There is little connection, or a very complex pattern, between the incidence of pancreatic cancer and measures of socio-economic factors (8,13). In a review of the epidemiological characteristics of patients with pancreatic cancer in USA Fitzgerald and Cubilla (17) did the following summing-up:

The typical patient with pancreatic cancer is an elderly, obese, black, catholic, male, alcoholic house-wife, a diabetic who smokes cigarettes excessively and whose ancestors migrated from Japan to the United States via Hawaii.

Demographic risk factors for pancreatic cancer are hence unspecific and give few guide-lines for defining risk groups for primary or secondary prevention. One can almost characterize the disease as being that common cancer which has very similar rates in subgroups regardless of how a given population is subdivided (13).

2.3.2. Bili-duodenal reflux into the pancreas

In his thesis Hjort in 1947 stated (18) that "the theory of pancreatic reflux seems to be supported by anatomic, physiopathologic, pathologico-anatomic and chemical evidence". It is not known whether carcinogens and/or their active metabolites reach the pancreas as a con-

sequence of reflux of bile and duodenal contents into the pancreatic duct or directly via the blood supply. However, as the majority of human pancreatic cancers develop in the head of the gland (3), and to a greater extent than what can be explained by the relative amount of ductal epithelium in the head and the body of the pancreas (19), reflux into the pancreatic duct has been suggested as an etiological factor (20). Bile has been suggested as a co-carcinogen in the development of cancer of the resected stomach (21), but there is virtually no information available regarding the presence of suspected pancreatic carcinogens and mutagens in the human bile, duodenal content or pancreatic juice (22). However, although reflux into the pancreatic duct is possible in man (18, 23) it is still not known to what extent it occurs under physiological conditions.

2.3.3. Non-dietary factors

Extensive comparison of vital statistical data on occupation and pancreatic cancer has given no proof that any occupational group has a divergent rate of pancreatic cancer in Sweden (24), Finland (25) or elsewhere (13, 26, 27).

In a recent Swedish study there was a significant correlation, although with only a $r = 0.59$ for males and $r = 0.40$ for females, between low dose radiation and pancreatic cancer (28), but other authors claim that the role of radiation has not yet been clearly established (8).

2.3.4 Smoking

Several studies (for reviews see 8, 13) have suggested that smokers suffer about a two-fold relative risk of developing pancreatic cancer. There has also been demonstrated a dose-response relationship for US male veterans (29). Furthermore, ex-smokers have an elevated risk, but less than active smokers (30). A 10-year follow-up study of 55 000 Swedish subjects aged 18-69 points to an increased mortality rate of 2.5 for female and 3.1 for male smokers in pancreatic cancer (13). Although this risk is much smaller than that of lung cancer, the mortality data on pancreatic and lung cancer are highly correlated in males, suggesting an association between tobacco and these two tumors (31). Whether this is casual or not can still be debated, but the generally accepted view is that cigarette smoking correlates with increased rates of pancreatic cancer and may be responsible for an important part of the increase in pancreatic cancer incidence in recent decades (13). Which component of tobacco smoke that is responsible for the carcinogenic effect is not known, but there have been speculations on the importance of the nitrosamines, arsenic, naphthylamines and nicotine in the smoke (32).

2.3.5. Sex

Of certain interest is the male/female ratio in pancreatic cancer. As can be seen in Fig. 2 the relative incidence rate for men and women is age-dependent; the difference in ratio is clearly decreasing 1-2 decades after menopause.

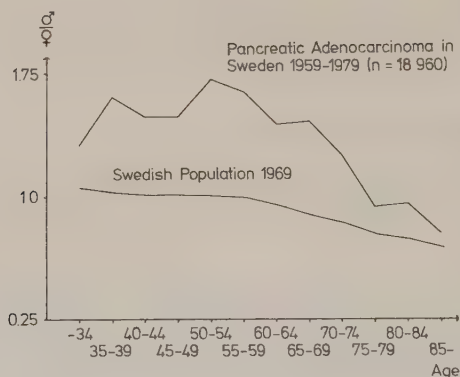


Fig 2. The male/female (M/F) ratio in patients with pancreatic cancer diagnosed in Sweden between 1959 and 1979 as compared to the M/F ratio of the whole Swedish population. The ratios of different age groups are given (Source: The Cancer Registry, National Board of Health and Welfare, Stockholm, Sweden).

In a clinical series von Heerden et al (33) found that the prognosis for women was more favourable than that for men. It has also been shown that there are estrogen receptors both in human healthy pancreas and in pancreatic cancer tissue (vide infra) which may be of importance in this respect.

2.3.6. Diabetes

A casual relationship between diabetes and pancreatic cancer has been proposed. Diabetic patients today survive longer than three or four decades ago, but still their observed death rates from all causes, including cancer, are significantly in excess of those of a non-diabetic population (34). In USA a correlation between diabetes morta-

lity rates and pancreatic cancer mortality is reported for women but not for men (35,36).

Among the 265 patients with carcinoma pancreatis investigated by Karmody and Kyle (37) 51 had overt diabetes, but 25 of them had both diagnoses made within a period of 3 months, which raises the question whether diabetes is a sign or a consequence of pancreatic cancer or if it is an etiologic factor. Perhaps some common underlying factor (genetic, viral etc) induces both diseases (8). The latter opinion is supported by the finding that patients with both diabetes and pancreatic carcinoma hardly ever have so large tumors that a reduction of the number of islet cells is the cause of diabetes (2). Furthermore, it has been assumed that altered glucose homeostasis or changed levels of islet cell hormones may be a promotor of a carcinogenic effect. It has also been shown experimentally that the peri-insular exocrine cells are more sensitive to carcinogens (nitrosamines) than the tele-insular ones, which might speak in favor of a paracrine effect of insulin or other islet cell hormones (38). Pour and co-workers found no nitrosamine-induced tumors in alloxan-treated Syrian golden hamsters and concluded (39) that islet cell function is essential for the carcinogenic function of the nitrosamines.

Against an association between diabetes and pancreatic cancer speaks an autopsy study of 448 patients with diabetes 50 years of age or younger (40) of whom only five had gastrointestinal cancer (this group of patients must include those with diabetes since childhood, who therefore had had an altered endocrine milieu for the exocrine cells during a long time).

In conclusion the co-variation between diabetes and pancreatic cancer is still unexplained and the only group of diabetics who possibly could benefit from screening for pancreatic cancer is non-obese patients who develop diabetes late in life (41).

2.3.7. Pancreatitis

Mikel and Campbell (42) reported on an autopsy study of 100 consecutive cases of carcinoma of the pancreas and found gross pancreatitis with fat necrosis in 23 %, microscopic evidence of pancreatitis in 49 % and pancreatic cysts in 15 %. However, two-thirds of the pancreatic carcinomas occurred in the head of the pancreas, which might indicate that the pancreatitis was secondary to obstruction of the pancreatic duct in most cases. This is further supported by Gambill (43) who found that significant pancreatitis (by histologic criteria) was present in 26 of 225 consecutive patients with pancreatic carcinoma, and one or more attacks of acute pancreatitis antedated abdominal operations for cancer in 31 % of these 26 patients. Some authors have claimed a high incidence of pancreatic cancer in patients with chronic pancreatitis and pancreatic calcification (44, 45) but on the other hand Johnson and Zintel (46) found no chronic pancreatitis among 133

patients with pancreatic cancer, and no pancreatic cancer in 64 patients with chronic pancreatitis. Besides, the clinical differentiation between pancreatic cancer and chronic pancreatitis can be extremely difficult, non-operatively as well as operatively (47).

In summary, while there in some cases is an association of pancreatic cancer with pancreatitis, especially chronic pancreatitis with calcification, there is little evidence to suggest this relationship to be other than random, or secondary to pancreatic duct obstruction, or an inflammatory response around a neoplastic lesion, and less reason to think it is casual (13).

2.3.8. Other diseases

An association between pancreatic cancer and bronchial asthma (48), rheumatoid arthritis, morbid obesity and multiple malignant neoplasm has been proposed, but no evidence for a causal relationship to any of those has been given (13).

2.3.9. Dietary factors

Recently some dietary factors have been considered of certain interest as possible causative factors for pancreatic cancer, especially fat, protein, coffee and alcohol. No clear relationship has been established between diet in general and pancreatic cancer, but Japanese data suggest a relationship to a high meat "western" diet (49). International pattern of pancreatic cancer correlates with several dietary variables including high dietary intake of fat and protein (50-52). This has also been investigated experimentally by Pour (38) who found that a protein-free diet inhibited the carcinogenic action of nitrosamines in the Syrian golden hamster (53).

2.3.10. Coffee

MacMahon and co-workers (54) proposed a close relationship between coffee-drinking and cancer of the pancreas: The relative risk associated with a consumption of up to 2 cups of coffee per day was 1.8 and with 3 or more cups per day 2.7. Since 1981 at least 11 papers on this association have been published (for references see 55-57) but none of them has verified the initial findings. Thus, available data do not favor any casual relationship between consumption of coffee and pancreatic cancer.

2.3.11. Alcohol

The association of chronic alcoholism and acute and chronic inflammation of the pancreas is well recognized (58). There are studies pointing to an increased risk for pancreatic cancer in patients with chronic alcoholism. In a study of 4 370 Finnish male misusers the observed number of pancreatic cancer exceeded the one expected, but

the difference was not significant (59). Other authors did not find any association (19, 24, 60). It is difficult to settle if this relationship is casual as alcohol abuse is associated with many confounding factors: association to chronic pancreatitis, coffee-consumption, smoking etc. In summary, while there are some reports on alcohol as a risk factor for pancreatic cancer, none of them provides any strong evidence (13).

2.4. Theoretical basis for hormonal influence on pancreatic carcinogenesis

2.4.1. Gastrointestinal hormones

The pancreatic secretory function is regulated by a complex interplay between neural and hormonal mechanisms and recently it has become evident that - at least - hormones also may have a trophic effect on the gland. This raises the question whether factors resulting in an excessive and sustained release of gastrointestinal hormones may influence the development of a pancreatic cancer, e.g. by sensitizing the gland to environmental carcinogens (61). The most important inductor of hormonal stimulation of the pancreas is diet. Johnson and co-workers (62) have shown that animals deprived of food but given calories via total parenteral nutrition developed atrophy of the pancreas. Mainz, Parks and Webster (63) reported that fasting for six days resulted in decreased DNA synthesis and content as well as reduced pancreatic weight, but refeeding resulted in a marked increase of DNA synthesis and a return of DNA content to almost normal levels after three days. Thus, fasting, or endogen hormonal deprivation, may result in a reversible atrophy of the pancreas.

It has also been made clear that long-term subcutaneous administration of cholecystokinin (CCK) and its octapeptide (64) have a trophic effect on the exocrine pancreas, and also an influence on glucose tolerance (64). Similar effects have not been shown after secretin administration, but the effect of CCK on enzyme secretion is potentiated by secretin (65). Moreover, it has also been shown that most of the gastrointestinal hormones influence enzyme secretion by the exocrine pancreas. Thus gastrin, bombesin and substance P are stimulatory, whereas PP, somatostatin, enkephalins and glucagon are inhibitory (66). Whether or not any of these hormones are released by the diet in such amount that they directly or indirectly exert a trophic effect of the human pancreas is still not known. The fact that people with chronic hypergastrinemia (e.g. patients with pernicious anemia and the Zollinger-Ellison syndrome) are known not to have an increased risk for pancreatic cancer could reflect an adaptive pancreatic response to sustained overproduction of gastrin: or it could reflect a greater importance of CCK in pancreatic carcinogenesis. So it may not necessarily be the effects of chronic, but rather of intermittent, repeated, gastrointestinal hormonal stimulation of the pancreas that is harmful (67).

2.4.2. Enzyme inhibitors

Pancreatic hypertrophy and hyperplasia have been demonstrated in chickens and rats after ingestion of uncooked soya beans (68), which contain a heat-labile trypsin inhibitor. Similar effects on the exocrine pancreas have also been reported after feeding with other trypsin inhibitors such as lima bean inhibitor, ovomucoid inhibitor, bovine trypsin inhibitor, para-amino-benzamidine and Trasylol^R. Probably these effects are caused by an endogen release of gastrointestinal hormones or factors (69, 70). It has also been observed that dietary fiber exerts an inhibitory effect on the activities on intraduodenal enzymes of pancreatic origin in vitro (71) and in vivo (72).

Long-term parenteral trypsin inhibitor administration did not result in changes of pancreatic morphology or enzyme activities in the rat (73), which suggests that the intestinal mucosa is involved in the mediation of the trypsin inhibitor-induced effects on the pancreas. On the other hand subcutaneous administration of CCK and long-term oral trypsin inhibitor resulted in similar, but not identical, changes of exocrine pancreatic weight and enzyme activities (74). This negative feedback system of intraluminal trypsin on the exocrine pancreas (75) is also operative in the pig (76), whereas there is still no agreement if the mechanism exists in the human (77-79).

There is also a report from England that points out soya rather than coffee as an explaining factor for the increase of pancreatic cancer 1948-78 (80).

2.4.3. Steroid hormones

It has been suggested that steroid hormones possess the ability to influence pancreatic function, and cortison treatment has been brought up as a cause, although unusual, of acute pancreatitis (81). Hormonal and hormone-linked treatment have been reported to be of value in malignant disease of the prostate (82, 83) and the breast (84). Sandberg & Rosenthal (85) have shown that both estrogen and androgen receptors exist in acinar cells of the pancreas in humans, rats, guinea-pigs and baboons. They have also stated (85), although no methodological details were given, that administration of estrogens to dogs changed the composition of the pancreatic juice: decreased the protein concentration and the levels of zink and bicarbonate and increased the activities of amylase and lipase. However, the high affinity, low capacity receptor for estrogen (ER) is quantitatively overshadowed by a high capacity estrogen-binding protein (EBP) (85), and Pousette et al (86) recently found this protein to constitute about 4 % of the total protein content of the cytosol of the human pancreas. Further, Greenway et al (87) have found ER in cytosolic and nuclear fractions of human pancreatic carcinoma and human fetal pancreas, making depression of fetal genes a possibility. They also reported on high activities of aromatase and 5- α -reductase and sug-

gested these findings to be consistent with the low levels of circulating testosterone found in both men and women (88). Satake et al (89) found ER in 7 of 16 DMBA-induced pancreatic carcinomas in the rat, whereas Molteni et al (90) found ER only in acinar adenocarcinoma of the rat but not in ductal adenocarcinoma of the Syrian golden hamster. Hayashi and Katayama (91) concluded, from experiments with a carcinogen known to induce acinar pancreatic carcinoma in the rat, that testosterone can promote the induction of pancreatic tumors in the rat.

Hence, there is sufficient evidence to propose further investigations with the aim to influence pancreatic cancer with steroid hormone manipulation, i.e. antiestrogen or antiandrogen therapy, or with cytostatic drugs coupled to a hormone causing a more selective accumulation of the drug within the cancer tissue. This latter technic has been used in estramustine phosphate (Estracyt^R), which is used in the treatment of prostatic carcinoma (83). This molecule is in vivo rapidly dephosphorylated to estramustine, which combines 17- β -estradiol and nornitrogen mustard via a carbamic ester (92). Future studies on estramustine in pancreatic cancer might be one way to derive benefit from the recent studies on sex-hormones and pancreatic carcinogenesis.

2.5 Therapeutic results - present status

2.5.1. Curative surgery

Attempted curative surgery in pancreatic cancer can be done as pancreatico-duodenectomy ad modum Whipple, total pancreatectomy or regional pancreatectomy ad modum Fortner. Whipple introduced his method in 1935 (93) and six years later reported on the results of 41 cases, on whom he himself had done the operation in 7 cases (94). Of the 41 patients 18 had exocrine pancreatic cancer - 8 of them died post-operatively. Mongé and co-workers in 1964 (95) reviewed 119 patients who had a Whipple operation for pancreatic tumors with a hospital mortality of 21 %, a 3-year survival of 24 % and a 5-year survival of 18 %. Of the patients surviving 5 years, however, 4 had tumors classified as islet cell carcinoma and the 5-year survival for ductal carcinoma of the pancreas was 14 %. From Sweden Bergstrand et al (96) reported on 44 cases of Whipple operations for exocrine pancreatic cancer with a 25 % hospital mortality and a median survival of 13 months, and Björck et al (97) found a 10 % hospital mortality and an 8 % 5-year survival in a series of 62 patients. Longmire collected - from six reports - (98) 599 cases of pancreatic cancer operated on according to Whipple and found an operative mortality between 10 and 25 % (median 21 %) and a 5-year survival between 3 and 8 % (median 7 %). In another study Björck et al (97) put together 1 029 cases from series published 1970-1979 and found a 5-year survival of 5 %.

The rationales for doing total pancreatectomy are the intraductal

spreading and the multicentricity of cancer, the better clearance of lymph nodes and the avoidance of a complication-prone anastomosis - the pancreatico-jejunostomy. Matusi et al (99) have shown that out of 18 total pancreatic specimens removed surgically there were 5 without lymph node involvement or extra-pancreatic invasion, in which the cancer proceeded per continuitatem to the tail along the pancreatic duct. Tryka and Brooks (100) showed that in 9 out of 25 specimens from total pancreatectomy tumor tissue would have been left in the remaining part of the pancreas if a Whipple procedure had been done instead of a total pancreatectomy, in five instances due to multifocal tumor growth. Cubilla, Fortner and Fitzgerald (101) found the average number of lymph nodes in surgical specimens after total pancreatectomy to be 41 and after Whipple operation 33. The most common cause of complications after a Whipple procedure is leakage from the pancreatico-jejunostomy (102).

The first successful total pancreatectomy was done by Priestly in 1942 (103), and a report on 6 cases with only one hospital death from the Mayo Clinics was given in 1948 (104). In 1954 it was possible to collect a series from the literature (105) of 102 patients who had undergone a total pancreatectomy with an operative mortality of 36 %. In a review of 64 total pancreatectomies for cancer performed at the Mayo Clinic between 1942 and 1973 it was concluded (106) that the results of the operation with 14 % operative mortality, 53 % survival after 1 year, and 25 % after 3 years, were comparable to those of standard Whipple procedures for malignant lesions. Among more recent materials is one reported by Herter et al (107) on 66 total pancreatectomies with a 25 % hospital mortality, a median survival of 6 months and a 5-year survival of 2 % (1 patient). Of certain interest is the report by Moossa (108) who found it possible to perform a total pancreatectomy in 24 patients referred to him after a laparotomy in other hospitals had shown "incurable pancreatic cancer".

The quality of life after a total pancreatectomy has been evaluated by Warren et al (109) and Toregard and Ihse (110), who stated that the diabetes and nutrition usually can be adequately managed and that the quality of life is good as long as the patients are free from recurrent disease.

The so called regional pancreatectomy described by Fortner (111) includes resection of the portal vein and sometimes the superior mesenteric artery, and in addition a meticulous lymphadenectomy. Hitherto, however, no improvement in long-term survival has been reported after such procedures and further the hospital mortality is high (112).

2.5.2. Palliative surgery

More than 80 % of all operations for pancreatic cancer are done for palliation (bilio-digestive shunting to relieve biliary stasis,

gastro-enteroanastomosis to eliminate gastric outlet obstruction and pain-relieving measures such as splanchicectomy, and splanchic block). In a series of 111 patients operated palliatively Brooks et al found a median survival of 6 months (113). Feduska et al (114) reported in a study of 101 patients on a 30-days' mortality of 41 % after a diagnostic and 33 % after a palliative operation, and a median survival of 4 months after diagnostic and 6 months after palliative operations. However, Crile (115), after studying 28 patients operated on according to Whipple and 28 operated on with by-pass, found that the latter operation in most cases was advantageous to patients (longer median survival, 12 vs 6 months, better 1-year survival, 32 vs 12 %, and longer maximal survival, 41 vs 22 months).

In a Swedish material (116) of 228 patients with pancreatic carcinoma treated in a small hospital during 30 years, the median survival of 4 months, and the hospital mortality of 18 %, remained unchanged during the three decades investigated.

2.5.3. Radiotherapy

The deep location of the pancreas and its intimate anatomic association with the bowel, liver, kidneys, stomach and spinal cord largely precluded the delivery of adequate doses of external beam irradiation prior to the megavoltage era, and cancer of the pancreas acquired a reputation of being "radio-resistant" (117). With new imaging technics, i.e. computerized tomography and ultrasonography, and by using radio-opaque clips for mapping out the tumor at laparotomy, it has been possible to increase the precision of the beams and therefore higher doses can be given. When giving 7 000 rads with this technic to 41 patients with histologically verified cancer the survival was found comparable to that of 31 patients operated on for cure with a 52 % 1-year and 9 % 3-year survival (118). The authors conclude that adenocarcinoma of the pancreas is not a radio-resistant neoplasm and may even in some cases be radio-curable.

Radiation therapy has also been carried out as interstitial radiation using radon seeds, but with only limited effect (119). Also a combination of external and intra-operative irradiation has been tried (120), a sophisticated but promising technic. Finally, in one study the combination of chemotherapy (5-fluoro-uracil) and radiation gave a significant improvement in survival (121).

2.5.4. Chemotherapy

The difficulties to evaluate the effect of chemotherapy in pancreatic cancer have been considerable, most of all due to the lack of measurable parameters. Lately ultrasonography and computerized tomography have been used to document changes in size of pancreatic tumors during treatment with chemotherapy, but the validity and sensitivity of these imaging technics are presently unknown. From a theoretical

point of view they are of doubtful value as morphology gives little possibility to distinguish viable from non-viable tissue, and cancer tissue from surrounding inflammation.

Compared to other gastrointestinal cancers, as for example gastric cancer and colo-rectal cancer, only few cytostatic drugs have been properly tested in pancreatic cancer. 5-Fluoro-uracil (5-FU) is the most often studied single drug, and had in a collection-series a response rate of 28 %, but was of little benefit in terms of patient survival or palliation of symptoms (122). The range of response rate in different trials is also considerable, partly due to different criterias used for evaluation of response, partly due to different ways of patient selection. Among other cytostatic drugs mitomycin C has proved to exert some effect with a response rate between 2 and 27 % (122) and streptozotocin, a drug generally used for treatment of endocrine pancreatic cancer, has given a response rate of up to 36 % in a small, multi-center material (8 of 22 patients) (122).

Combination chemotherapy has given a somewhat higher response rate. In a study of Adriamycin^R, mitomycin C and 5-FU (123) there was a response rate of 37 % (10/27 evaluable patients) with a median survival of 6 months. Mallinson et al (124) using 5-FU, cyclophosphamid, vincristine and methotrexate at the start of therapy, and 5-FU and mitomycin C at six weekly intervals increased median survival from three to eleven months. In a Danish study (125) patients given 5-FU and BCNU did not benefit as compared to a control group given placebo. We have used 5-FU, vincristine and CCNU in a controlled, prospective study (126) without finding any beneficial effects - the complication rate was however significant.

In summary there are today no modalities of chemotherapy suitable for routine use in pancreatic cancer, and the experiences from ongoing research do not give a great hope of a break-through in a near future.

2.6. Experimental models

2.6.1. Acinar pancreatic cancer

Exocrine pancreatic cancer was first induced by Wilson et al (127) in 1941 who found acinar cell changes in 17 of the 29 albino rats given 2-acetylaminofluorene in the food during 103 to 133 days. One of the animals had an acinar cell carcinoma of the pancreas, but there were also tumors in the urinary bladder, renal pelvis, liver and lungs. Acinar cell tumors were also found by Hoch-Ligeti (128) in 3 of 50 Wistar rats given p-dimethylaminoazobenzene in the food during 15 months. Acinar adenomas were also found by Morris and Eyestone (129) in a dog given 2-acetylaminofluorene in the diet.

A high tumor incidence with neoplasms of acinar appearance was first reported in 1971 by Hayashi and Hasegawa (130) after intravenous

administration of 4-hydroxyaminoquinoline-1-oxide (4-HAQO) to rats. Azaserine is yet another compound capable to induce acinar cell carcinoma (131). The combination of azaserine and 4-HAQO potentiated each other's action (132).

At bioassays of more than 200 chemicals the National Cancer Institute's carcinogenesis testing program in USA (133) found only two to be pancreatic carcinogens.

2.6.2. Nitrosamine-induced cancer

The first successful induction of pancreatic tumors of ductal/ductular origin was reported by Druckrey et al in 1968 (134). Methylnitrosourea and methylnitrosourethan administered in the drinking water gave, beyond the scope of the experiment, pancreatic adenocarcinoma in 2/26 and 14/38 of the guinea-pigs, respectively. In similar studies with nitrosamines in the Syrian golden hamster - studies aiming at induction of tumors of the respiratory tract - Pour et al (135) in 1974 found pancreatic carcinomas in a high frequency after a short induction time. The neoplasms closely resembled to human pancreatic cancer morphologically as well as biologically: they were multifocal and metastasized to lymph nodes, liver and lungs. Initially N-nitrosobis(2-hydroxypropyl)amine (BHP) (Fig. 3) was used, but as this compound also gave primary cancer of the respiratory system, liver, gall-bladder and kidney (38), it has largely been replaced by N-nitrosobis(2-oxypropyl)amine (BOP) (Fig. 3), which in equitoxic doses induces considerably less extrapancreatic cancer (no tumors of nasal cavity, larynx or trachea, and few cancers of lung, liver, gallbladder and kidney) (38). Further studies have suggested that N-nitroso(2-hydroxypropyl)(2-oxypropyl)amine (HPOP) (Fig. 3) may be a proximate pancreatic carcinogen after administration of both BHP and BOP (38).

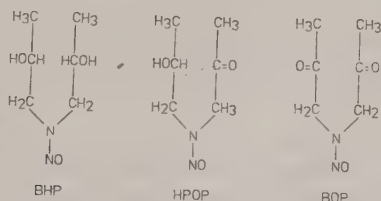


Fig 3. Molecular structures of pancreatic carcinogens in Syrian golden hamsters.

HPOP can exist as a tautomeric mixture of the open chain and cyclic forms, which is of certain interest as the cyclic form is structurally related to both hexose sugars and to the sugar-moiety of streptozotocin (Fig. 4) - well known to interact strongly with the endocrine pancreas. Unfortunately HPOP is unstable and not suitable for routine animal work (38).

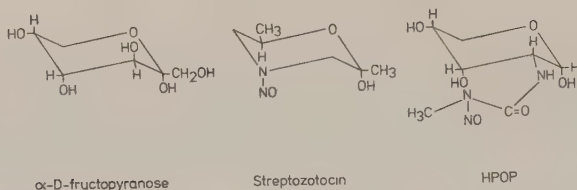


Fig 4. Cyclic hemiacetal form of HPOP in comparison to the structures of hexose sugar and to the sugar moiety of streptozotocin.

It has been shown that HPOP is a metabolite from BHP as well as BOP, and HPOP has been isolated in both urine and blood after administration of BHP or BOP (38). There is a dose-response relationship of BHP as well as BOP. The carcinogenic effect of BOP is about 50 times stronger than that of BHP. After subcutaneous weekly injection of 2.5 mg BOP per kg bw or 125 mg BHP per kg bw to Syrian golden hamsters Pour et al (38) found a frequency of cancer-bearing animals of 88 and 95 % after 19 and 30 weeks, respectively. A single dose of BOP, from 2.5 to 40 mg per kg bw, gave a frequency of tumor-bearing animals from 7 to 62 % after a latency of 35 to 56 weeks, but after a single dose of 10 mg per kg bw or more there were also found tumors of the lung, liver, gall-bladder and kidneys, although the frequency was less than 10 % (38).

According to Pour et al (136) all neoplasms were found to have their origin in the ductal system. This was supported by the finding that hyperplasia and dysplasia of the ducts often preceded neoplasia. Small tumors remained localized to the gland for a relatively long time, which may be due to the obvious immunologically based lymphoplasmatic cell reaction around the tumors. However, finally they metastasize to lymph nodes, liver and lung (38).

The tumors can be transplanted to other Syrian golden hamsters. A progressively increasing rate of successful transplantation (137) with each generation from 60 to 100 % has been reported.

3. CHAPTER II: AIMS, MATERIALS, METHODS AND EXPERIMENTAL DESIGN

3.1. Aims of the present study

Pancreatic cancer continues to be a problem hard to solve. During the last years an increasing interest in pancreatic cancer research is obvious all over the world. The aim of the present thesis was to elucidate and define some prognostic factors with special emphasis on hormonal influences.

In the first part an analysis of a clinical material was done in order to create a background for the subsequent experimental studies. A group of patients operated on with attempted radical surgery was chosen to offer a material as uniform as possible.

In the first experimental study the usefulness and reliability of a model for induction of ductal pancreatic cancer in the Syrian golden hamster was evaluated. Changes in exocrine pancreatic secretion and in pancreatic digestive and lysosomal enzymes during experimental carcinogenesis was investigated. The model was also used to test the bile reflux theory in the pathogenesis of pancreatic cancer. The possible role of gastrointestinal hormones - administered exogenously or released endogenously by oral trypsin inhibitors - in the development of pancreatic cancer was then studied. This series of experiments was completed by studies on the presence and possible importance of steroid binding proteins in normal and cancerous pancreatic tissue.

3.2. Materials and methods

3.2.1. Clinical study

For more than 20 years total pancreatectomy has been the routine method at the Department of Surgery, University of Lund, when radical surgery has been attempted in patients with pancreatic cancer. During the years the criteria for selection of the patients have varied, the preoperative work-up and postoperative management of the patients have changed and a great number of surgeons have been involved in the operations. We have analyzed a series of patients in order to find factors which may be involved in the outcome of the disease. Altogether 41 variables were considered: 15 related to the patient, 5 related to the tumor and 21 related to the management.

In the majority of patients the operation was done with removal en bloc of the pancreas, great omentum, duodenum, common bile duct, gall-bladder, spleen and at least 50 % of the stomach.

The patients were staged according to a modification of the classification described by Hermreck et al (138):

- Stage I: The tumor is localized within the pancreatic capsule.
- Stage II: The tumor invades the duodenum.
- Stage III: Invasion of the superior mesenteric or portal vessels
 or regional lymph nodes.
- Stage IV: Distant spread of the malignant process.

Patients who died without leaving the hospital after the operation were considered as hospital deaths.

The statistical analyses were done by using chi-square test with Yate's correction when comparing two factors, chi-square test for trends in proportions when comparing three factors and generalized Wilcoxon test when comparing factors affecting long-term survival.

2.3.2. Experimental studies

2.3.2.1. Chemicals

The carcinogens, N-nitrosobis(2-hydroxypropyl)amine (BHP) and N-nitrosobis(2-oxypropyl)amine (BOP) were purchased from Ash Stevens Inc., Detroit, Michigan, USA.

Porcine crystalline trypsin was obtained from A/S Novo, Copenhagen, Denmark, and highly purified bovine lung trypsin inhibitor (Trasylol^R) was a gift from Bayer AG, Leverkusen, BRD.

Bile and bile-pancreatic secretions used for infusions were collected on ice from hamsters provided with bile duct or bile-pancreatic duct fistulae and immediately frozen at -20°C. The bile used was found to be free from trypsin or trypsinogen.

Cholecystokinin, secretin and somatostatin were obtained from Kabi-Vitrum, Stockholm, Sweden. Cerulein (Ceruletide^R) was kindly donated by Pharmitalia Research Laboratories, Milano, Italy.

Polyestradiolphosphate (Estradurin^R) was delivered by AB Leo, Helsingborg, Sweden, and double-labeled ³H, ¹⁴C-estramustine was synthesized for our disposal by the Research Laboratories of the same company. ³H-estradiol was obtained from New England Nuclear Chemicals GbmH, Dreieichenhain, BRD.

2.3.2.2. Laboratory technics

Protein was determined according to the method described by Lowry et al (139). Amylase activity was assayed using the Phadebas test, a gift from Pharmacia, Uppsala, Sweden. Bicarbonate was indirectly determined from pH and pCO₂ (140).

When determining the digestive and lysosomal enzymes in the pancreas the tissues were homogenized in ice-cold distilled and deionized water (1/100), β -hexosaminidase and α -mannosidase were then analyzed as described by Hultberg et al (141), acid phosphatase as follows: 50 μ l of 1 mmol/l solution of 4-methylumbelliferyl-phosphate were incubated for 30 minutes with 50 μ l 1 mmol/l citrate buffer pH 5.0 and 25 μ l homogenate, and β -glucuronidase: 100 μ l of 1 mmol/l solution of 4-methylumbelliferyl- β -D-glucuronide in 100 mmol/l citrate-phosphate buffer pH 4.5 were incubated for 30 minutes with 25 μ l homogenate. The reaction both for assay of acid phosphatase and β -glucuronidase was stopped by addition of 3 ml of 0.2 mol/l glycine buffer pH 10.7. The fluorescence of 4-methylumbelliferone was read as for β -hexosaminidase (141).

Estrogen receptors (ER) were measured with isoelectric focusing on polyacrylamide gel (142) and progesterone receptors (PgR) with the dextran coated charcoal method and Scatchard analysis (142). Estradiol-binding protein (EBP) was studied as ER but over a wider pH-gradient, 3.5-10 instead of 5.0-7.5. The concentration of ER, PgR and EBP was expressed as specifically bound 3 H-estradiol or 3 H-R5020 (PgR) per cytosol protein.

2.3.2.3. Animals and diets

Eight to ten weeks old Syrian golden hamsters from own breeding were used. Their average weight at the start of the experiments was about 100 g. The sexes were equally represented in all experiments, if not otherwise stated. During the long-term experiment the hamsters were housed in groups of two in plastic cages. In most experiments the animals were kept on a standard pellet diet (composed according to actual international specifications and containing the following raw material: fish protein concentrate, soya meal (roasted), fodder yeast, cereals, wheat sprouts, animal fat, soya oil, vitamin concentrate and minerals, delivered by Anticimex AB, Stockholm, Sweden), with free access to tap water. In one experiment a group of hamsters was kept on a diet of raw soya bean flour, supplemented with vitamins and minerals, Diasoy^R, and another group on heated soya bean flour, Soyolk^R (Special Diet Services Ltd., Essex, England).

3.2.2.4. Histology

Necropsy was performed according to a standardized schedule. After removal of the pancreas it was freed from fat and connective tissue, weighed and fixed in 10 % buffered formalin and embedded in paraffin. An average of 8-10 step sections were prepared from the gastric and splenic lobes of the pancreas and 2-4 from the duodenal lobe, which means that the distance between each section was at most 1.5 mm. The histologic preparations were stained with haematoxylin-eosin or erythrosin. The diagnostic criteria were in accordance with those used by Pour and Wilson (38). Ductal proliferation was classified as slight

(1-2 proliferations per low power field, 25 x magnification), moderate (2-4 proliferations per magnification field) or marked (>4 proliferations per magnification field). If the anatomic location of the histologic lesions was of importance their exact location was recorded on a diagram of the pancreas (Fig. 5).

The pathologist made all histological examinations without knowledge of the treatment given to the animal.

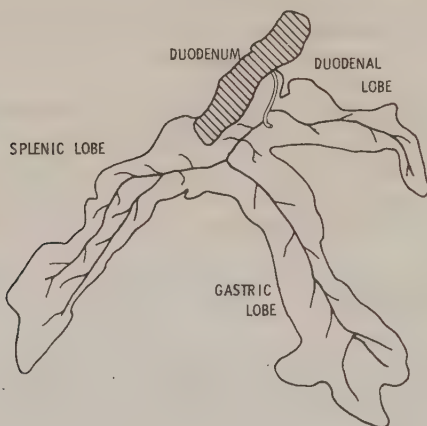


Fig. 5. Schematic diagram of the hamster pancreas.

3.2.2.5. Surgical procedures

The operative procedures were done under ether anesthesia or narcosis with intraperitoneal Mebumalum (ACO, Stockholm, Sweden). At the end of all operations 5-10 ml of a solution containing one part Ringer's and one part 5.5 % glucose solution was deposited s.c. in each animal.

Procedure used in the study of the effect of bile-deviation on pancreatic carcinogenesis. To preclude bile reflux into the pancreatic ducts a short segment of the bile ducts was resected just below the entrance of the cystic duct and a cholecystoduodenostomy was created at least 15 mm below the entrance of the bile duct into the duodenum. Control hamsters underwent a sham operation comprising thorough palpation and inspection of all intraabdominal organs.

Procedure used in the studies of pancreatic secretion. To collect bile-pancreatic juice a silastic catheter with an inner diameter of 0.58 mm and a length of 15 cm (Dow Corning 602-105) was introduced directly into the bile-pancreatic duct close to the duodenum and secured in place with a silk ligature. The tip of the catheter was placed at most 3 mm from the duodenal wall. When only bile was collected a catheter of similar type was introduced into the bile duct high up in the hilum of the liver. To make duodenal infusions possible another catheter of the same type was inserted into the duodenum just where the bile-pancreatic duct enters the intestine. Both catheters were brought out through separate stab incisions in the loin.

3.2.2.6. Calculations

Student's t-test for paired observations or the chi-square test was used in the infusion studies and Fischer's exact test when calculating the results of the long-term experiment. In the study of the effect of bilio-digestive by-pass of the pancreas the Log-Odd ratio test was used.

3.2.2.7. Studies on the sequential development of pancreatic cancer

In order to investigate the importance of the carcinogen dose and of induction time for the development of pre-cancerous and cancerous lesions a separate experiment was designed. This was also done in an attempt to elucidate if the carcinogen-induced changes in the hamster pancreas at any time after the start of the induction would show signs of regression with regard to frequency and morphology. The design of the experiment is shown in Fig. 6.

At the end of the experiment surviving animals were killed and standardized autopsies were done and histological specimens prepared as described above. (The results of the histological examinations were compared both between the animals in groups A, B and C given comparable doses, and within the groups given different doses.)

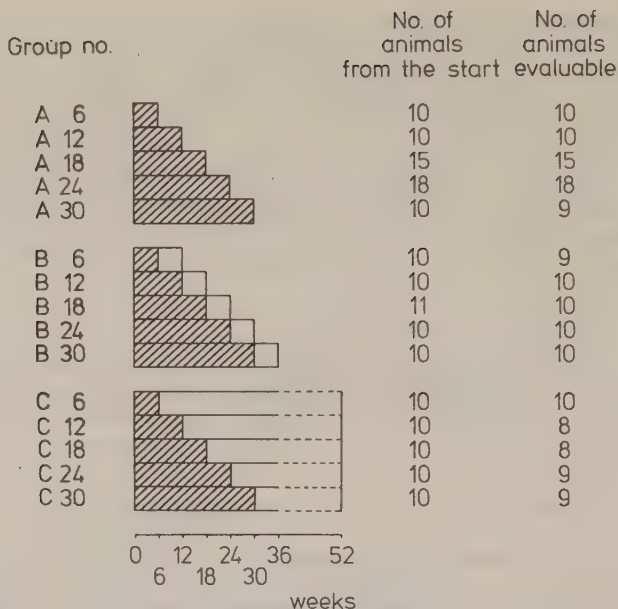


Fig 6. Design of the study of the sequential development of pancreatic cancer. The bars marked with lines represent weeks when BHP was given, the unmarked bars weeks when no carcinogen was given.

3.2.2.8. Studies on the pattern of pancreatic lysosomal enzymes during pancreatic carcinogenesis

A divergent change of lysosomal and digestive enzyme activities, respectively, in pancreatic secretion has been shown in hamsters with BOP-induced pancreatic cancer. The influence of BOP on the enzyme pattern within pancreatic tissue is unknown. Therefore, four different lysosomal enzymes (vide supra) and amylase were determined in pancreatic tissue of hamsters given weekly subcutaneous injections of BOP (10 mg/kg bw) for 6, 12 and 18 weeks.

3.2.2.9. Studies on pancreatic secretion

Animals in which secretory studies were to be done were operated on as described above, and kept in restraining cages. They were allowed about 3 hours of recovery after surgery before the start of the experiments, all of which were performed on conscious animals. If sur-

gical complications occurred the animals were excluded from the study. Food and water were withdrawn. In all experiments bile-pancreatic secretion was studied in one-hour samples.

In the study of influence of BOP on pancreatic secretion the spontaneous bile-pancreatic secretion was collected for 3 hours and then for another 7 hours after different doses (10 or 100 mg/kg bw) of BOP i.v. or s.c. Two groups of hamsters were pre-treated with 10 mg BOP/kg bw s.c. weekly for 6 or 12 weeks and investigated as above before and after administration of 10 mg BOP/kg bw s.c. Control hamsters were given 0.9 % saline s.c. Bile secretion was studied after injection of 10 mg BOP/kg bw.

In the study of feed-back regulation of pancreatic secretion the spontaneous secretion was initially studied during 14 hours to evaluate the influence of time and loss of fluid on secreted volume and protein output. Then the influence of intestinal bicarbonate (0.05 M) infusion (0.5 ml/h) and re-introduction of bile (0.5 ml/h) into the intestine was investigated. Bile-pancreatic juice was re-introduced into the duodenum without (0.5 and 1.0 ml/h) and with trypsin inhibitor (Trasylol^R 300 000 Kallikrein Inhibitory Units (KIU) corresponding to 50 000 KIU/ml) added to the infusates (0.5 ml/h) 3 hours after the start of the bile-pancreatic juice infusions. Finally trypsin (2.5 mg/ml) was infused (0.5 ml/h) into the duodenum without and with trypsin inhibitor in a dosage as described above.

3.2.2.10. Studies on hormonal influence on pancreatic enzyme content

In a series of ten-days experiments groups of hamsters were given cholecystokinin, secretin or somatostatin or, as a control, 0.9 % saline s.c. twice daily. Another group of hamsters were given a single dose of polyestradiolphosphate and the last group was given 50 000 KIU trypsin inhibitor in 0.5 ml of saline by gastric intubation twice daily. After sacrifice the pancreas was homogenized and its protein and amylase content were determined.

3.2.2.11. Studies on hormonal influence on pancreatic carcinogenesis

Pancreatic cancers were induced by s.c. injection of 125 or 250 mg of BHP or 10 mg of BOP per kg bw weekly for 6-24 weeks. The hormones were dissolved in 0.9 % saline and given as s.c. injections twice daily for five days a week during the same time as BHP and BOP. Two µg of cerulein was given in each injection, 2 IDU of cholecystokinin, 2 CU of secretin. The same doses were used when cholecystokinin and secretin were combined. Indirect hormonal stimulation was achieved by feeding animals with trypsin inhibitor (raw soya bean flour, Diasoyl^R) ad libitum. Control animals were fed heated soya bean flour, Soyolk^R.

Pancreatic wet weight was used as a measure of trophic hormone action on the gland. The influence on carcinogenesis was evaluated by

counting the number of (tumor)cancer-bearing animals, the total number of (tumors)cancers, the number of (tumors)cancers per (tumor)cancer-bearing animal, the distribution of (tumors)cancers, the frequency of pre-neoplastic lesions and the histological appearance of the induced benign and malignant lesions.

3.2.2.12. Studies on estrogen-receptors, progesterone-receptors and estrogen-binding protein

The presence of estrogen-receptors (ER), progesterone-receptors (PgR), and estrogen-binding protein (EBP) was studied in normal pancreas and BOP-induced pancreatic cancer in the hamster, and, for comparison, in liver tissue. Further, ER, PgR and EBP were measured in one sample of normal pancreatic tissue from cow, sheep, pig, guinea-pig, rat and mouse, respectively.

To investigate if long-term administration of estrogen influenced the hormone-binding capacity of the pancreas, glands from four hamsters given seven monthly polyestradiolphosphate injections were investigated for the presence of ER, EBP and PgR.

3.2.2.13. Studies on estramustine

The presence of estrogen-receptors (ER) and an estrogen-binding protein (EBP) has been demonstrated in normal human pancreatic tissue and ER in human pancreatic cancer. Estramustine phosphate, in vivo rapidly dephosphorylated to estramustine, is a molecule combining 17- β -estradiol and nor-nitrogen mustard, and has been shown to have a direct cytotoxic activity in a human prostatic cancer cell line and is widely used in the treatment of prostatic carcinoma. In the first part of a series of experiments on estramustine phosphate in pancreatic cancer the uptake and binding of estramustine to normal and cancerous pancreas was investigated in the Syrian golden hamster.

Double-labeled estramustine was injected intravenously and at various times after dosing, 15 min, 1, 4, 8 and 24 h, the animals were killed by exsanguination and immediately possible pancreatic tumors, the rest of the pancreas, the liver, the kidneys and hamstring muscles were removed, weighed and the radioactivity was determined. The area under the tissue concentration curve, AUC, was calculated for each organ and isotope.

In an in vitro study, cytosols prepared from pancreatic tissues (normal pancreas from untreated hamsters, pancreas without macroscopical tumors, in BOP-treated hamsters, and pancreatic tumors) were incubated with 3 H-estramustine in the absence or presence of a thousand-fold excess of unlabeled compound. The protein-bound radioactivity was obtained by gel filtration through small Sephadex columns. Aliquots of the protein-bound radioactivity were loaded onto a linear sucrose

gradient and centrifuged for 23 h. The gradients were analyzed for absorbance at 280 nm and fractions of about 80 μ l were collected and counted for radioactivity.

4. CHAPTER III: RESULTS AND DISCUSSION

4.1. Evaluation of prognostic factors in patients with pancreatic cancer operated on with total pancreatectomy (I)

A retrospective analysis of factors influencing short-term or long-term survival after total pancreatectomy for pancreatic cancer was done in 86 patients. In total 41 factors were considered.

Hospital mortality is generally defined as death within the first 30 days after the surgical procedure, but it seems more adequate to use the definition death after the operation without leaving the hospital, especially as modern intensive care not infrequently prolongs life also in desperate cases. Thus, hospital mortality was 21 % (18/86) and 29 % (25/86) in the present study when the two definitions were used, respectively.

Of all patients suffering hospital mortality (not leaving the hospital after surgery) 76 % were men, implying 40 % of all men, compared to 18 % of the women. The mean age did not differ between patients in the hospital mortality group and those who survived the operation. However, when separately analyzing patients 70 years of age or more it was found that 7/12 (58 %) did not survive the operation - although they at assessment preoperatively were considered fit for their age - compared to 18/69 (24 %) for patients younger than 70, a statistically significant difference. Previous or concomitant disease did not influence hospital mortality except for the presence of preoperative diabetes. Thus, 7/25 of the patients who suffered hospital mortality were diabetic as compared to 5/61 among those who survived the operation. Abdominal pain was the only presenting symptom which was predictive; 11/25 patients in the hospital mortality group had abdominal pain as the first symptom compared to 11/61 of the survivors.

The estimated size of the tumor did not influence hospital mortality, nor did the degree of histological differentiation have any bearing on the short-time prognosis. The stage of the tumor was of importance. There was a significantly lower hospital mortality in patients in stage I and II as compared to stage III. There was also a better chance for patients without increased S-bilirubin on the day of the operation, and those with preoperative bile-drainage. Also prophylactic antibiotic treatment reduced hospital mortality. Compared to other surgeons those who did 10 or more operations experienced a significantly lower hospital mortality (10 %).

Concerning factors influencing long-term survival only the stage of the disease was of importance (Fig. 6). Age did not influence long-term prognosis, and the five oldest patients surviving the operation, 71-78 years of age, had a survival life-time of 16 months (median 19 months), which is to be compared to 14 months in patients younger than 70 years of age at operation. The one-year survival was 25 % for men

and 46 % for women, and all 4 patients surviving 72 months were women (all males were dead by 36 months postoperatively). The tumor size in this selected material was without prognostic significance, as was the degree of differentiation.

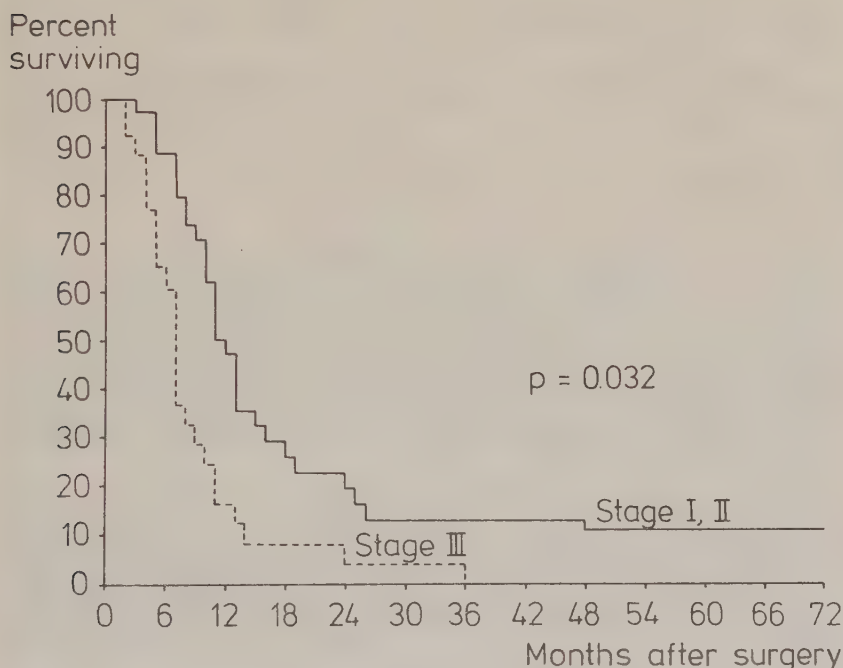


Fig 7. Influence of stage of the tumor on long-term survival (hospital deaths excluded) after total pancreatectomy for pancreatic cancer.

Among the 61 patients surviving the operation 57 died from or with recurrence of the disease. One patient lived for 17 years and had no signs of recurrence at autopsy but died from chronic pyelonephritis.

One patient died in 1961 five weeks after she had left the hospital in good condition, two months postoperatively, in a lethal attack of hypoglycemia.

The overall results in this series were less favorable than those of Herter et al (107), Brooks & Culebras (143), and Pliam & ReMine (106) who reported on a hospital mortality of 23, 13, and 14 %, respectively, and a mean survival of 21, 23 and 22 months, respectively. However, these series are fairly recent, and only few and experienced surgeons were involved, which is to be compared to a number of 25 surgeons performing the operations in our 23-year material.

Put against the backdrop of this gloomy long-term survival after attempted radical surgery for pancreatic carcinoma hospital mortality must be reasonably low in order to warrant the use of such an operation and the key-word for the efforts to reduce hospital mortality after pancreatectomy seems to be selection: selection of the patient, selection of the tumor and selection of the surgeon.

As mentioned above the factors that appeared to influence hospital mortality in the present study were age of 70 years or more, pre-operative diabetes, pain as presenting symptom, increased S-bilirubin at operation, preoperative bile drainage, prophylactic antibiotic treatment, stage of the tumor and experience of the surgeon. All of these factors can be taken into account preoperatively, either for guidance of selection of the patient, or for the planning of peri-operative management. Thus, it seems evident that hospital mortality can be kept on a reasonably low level if due regard is paid to failures done in the past.

Long-term survival was found to be influenced only by sex of the patient and stage of the tumor - factors difficult to affect. As death was associated with hypoglycemia in two of the patients, however, careful management of the diabetes seems to be important. Both patients dying of hypoglycemia succumbed before this complication was enough paid attention to, and today management of the postoperative diabetes seems to be more safe.

In summary, the results of treatment of pancreatic cancer by pancreatectomy imply that factors related to hospital mortality should be possible to control, whereas factors related to long-term prognosis are difficult or impossible to affect. The improvement of long-term survival must await means of an earlier identification of the disease and development of better adjunctive treatments. A prerequisite for the latter is an improved knowledge of the pathophysiology of pancreatic carcinoma - knowledge that can probably be acquired mainly by experimental studies.

4.2. Sequential development of pancreatic cancer in the hamster model (II)

An ideal experimental model for induced carcinoma shall: 1. resemble the human tumor biologically, 2. resemble the human tumor histologically, 3. be highly reproducible, 4. have a short latency time, 5.

give a high yield of tumors, 6. be dose- and time-dependent, and 7. require such a low amount of carcinogen that the animals do not suffer general toxicity. In the present hamster model the development of ductal and ductular proliferations, the number of tumor- and cancer-bearing animals, the number of tumors and cancers, the number of tumors and cancers per tumor- and cancer-bearing animals and the degree of differentiation of cancers were found to be related to the accumulated dose of the carcinogen. The duration of the period between the carcinogen administration and examination (spontaneous death or sacrifice) was another factor of importance for the development of the same changes except for the degree of differentiation of malignant tumors.

It seems clear from the results that once the neoplastic process has started it continues without signs of regression. The speed of the process can however be influenced by altering the dosage of the carcinogen, and the tumor yield can be controlled by choosing a longer or shorter duration of the experiments. Also, the degree of differentiation of the malignant tumors can be influenced by choosing different accumulated amounts of carcinogen administered. In our study, giving at least 6 weekly injections of BHP, we did not find any signs of regression of induced lesions. It is, however, still possible that induced pre-neoplastic changes may heal spontaneously, if e.g. lesser amounts of nitrosamines or single doses are used.

However, early ductal changes, such as dilatation and proliferation of ducts and ductules were frequently found even after small doses of carcinogens and short latency times. Therefore, subtle regressive lesions are probably not detected at light microscopy. On the other hand early cellular changes after a single dose of nitrosamines have been found at electron-microscopy (144) supporting the usefulness of that technic in studies of early carcinogenesis.

Our studies and those by Pour et al (38, 145), Rückert et al (146) and Sayers & Orloff (147) *inter alia* showed a different tumor- and cancer-yield, although the carcinogen, the dosage and time-schedule were comparable. Since the histological criteria were similar the differences found may be assigned to different biological susceptibility of hamsters from different colonies, or different strength of the carcinogen (i.e. due to different purity of the compound or due to effects of the handling). This underlines that each laboratory working with this model needs a reference system of its own and that adequate control groups always must be used.

The delivery of the model for experimental pancreatic cancer in the Syrian golden hamster - as used in our laboratory - was possible to modulate by changing carcinogen dose and induction time. The induced tumors resembled the human cancer histologically as well as biologically (38), and displayed a short latency time. A high yield of tumors was achieved, and the amounts of carcinogen needed did not

cause signs of general toxicity. Therefore - provided control groups are used - we find the model suitable as well as reliable for studies of other factors thought to be modulators of tumor development.

4.3. Influence of nitrosamines on pancreatic secretion and enzymes (III, IV)

In the present thesis the parameters hitherto used to evaluate effects of possible promoters of cancer development all have been based on histological criteria. In two experiments, however, we investigated changes in pancreatic secretion and enzyme content during carcinogenesis, induced by BOP, in the search for other useful parameters of the carcinogenic process.

In the first experiment 10 mg BOP per kg bw for 6, 12 and 18 weeks, respectively, caused tumor-bearing animals in a frequency of 25 %, 83 % and 100 %, and a cancer-bearing frequency of 0 %, 25 % and 83 % of the animals. The activities of α -mannosidase and acid phosphatase in the pancreatic tissue were uninfluenced by administration of the carcinogen. By contrast the activities of β -hexosaminidase and β -glucuronidase in pancreatic tissue exhibited remarkably high levels especially at 12 and 18 weeks. It was further obvious that the levels of pancreatic lysosomal enzymes in the control group did not change with time. A decrease of the specific pancreatic amylase activity was obvious after 18 weeks of carcinogen administration but not after 6 and 12 weeks. Also, a statistically significant fall by 20 % in pancreatic protein concentration was found after 18 weeks. When calculating ratios of lysosomal enzymes to amylase the diametrically opposite effect of the carcinogen on β -hexosaminidase and β -glucuronidase on one hand and amylase on the other was even more obvious. Also the ratio of α -mannosidase to amylase was statistically increased in the carcinogen group as compared to the controls after 18 weeks.

In the second experiment the acute effects of BOP on unstimulated exocrine pancreatic secretion were studied. Due to the minute amounts of juice obtained when pure pancreatic juice was collected combined bile-pancreatic juice was sampled. Both a single subcutaneous (10 mg/kg bw or 100 mg/kg bw) and a single intravenous (10 mg/kg bw) injection of BOP caused a reduction in secretory volume, and protein and amylase output from the bile-pancreatic fistulae. Also in animals pre-treated with weekly BOP injections for 6 or 12 weeks, a similar decrease in secretory volume and protein output following BOP administration was found. Bicarbonate output was assayed after a single subcutaneous BOP injection and in animals pre-treated with BOP for 12 weeks. Contrary to the BOP effects on secretory volume and protein output a clear-cut gradual increase in bicarbonate concentration was found after the BOP injection, which implies that the bicarbonate output was practically unaffected.

Reber et al (148) have reported a decrease in flow rate, digestive

enzymes and bicarbonate secretion after administration of another carcinogen, BHP, and considered these changes to be indicators of the malignant process. However, although their findings were dose- and time-dependent, the changes might as well reflect a depressive action of the carcinogen on pancreatic function, which not necessarily is related to carcinogenesis per se. In extended studies Doria, Mosley & Reber (149) investigated the acute effects of a single exposure of BHP given intravenously. They reported successful assays of not only protein but also bicarbonate and chloride in secretin- and CCK-stimulated pure pancreatic juice samples of 20 μ l. They found that the bicarbonate concentration was increased as early as 1 hour after administration of the carcinogen, and that the increase persisted for 2 weeks. Further, the protein concentration and output were depressed within 1 hour but increased significantly after 24 hours - an increase which persisted for at least 8 weeks. We have tried to use a similar experimental model design without success, because of highly varying and often minute samples of a viscous pancreatic juice - even at longer collection periods.

Rinderknecht et al (150) have recently reported pancreatic secretory abnormalities in hamsters given the same pancreatic carcinogen as used in the present study. They found a striking decrease in flow rate and decreased output of trypsinogen and trypsin whereas the output of β -glucuronidase and β -glucosidase in pancreatic juice increased markedly - changes that were observed before the appearance of histologically recognizable pancreatic tumors. Their findings are confirmed and extended by the present study. The origin of acid hydrolases in pancreatic juice and pancreatic tissue is not known at present, although indirect evidence suggests that at least some of them may originate from ductular as well as acinar cells (151), but may possibly also be produced by the tumor tissue itself (152). The most probable explanation, however, is that the inflammatory processes associated with the carcinogen administration are responsible for the increase in pancreatic lysosomal enzymes, as it is known that activated macrophages in inflammatory lesions secrete excessive amounts of lysosomal enzymes (153).

The results of the present study point to early changes, especially of the acinar cell function after administration of BOP, even if the decrease in secretory flow may reflect duct cell disturbances as well. As the alterations in protein (enzyme) secretion as well as pancreatic flow in our study occurred as early as after 1-2 hours a direct effect of the carcinogen on the pancreatic cells is most probable. This is consistent with the morphological findings of Levitt et al (154), who reported unspecific ultrastructural changes of both acinar and ductal cells 4 hours after the administration of BHP, and Ogrowsky & Wilson (144), who found similar changes as early as 45 minutes after a BOP injection. However, in long-term studies Flaks et al (155) found the dominant process observed after weekly administrations of BHP to be a dedifferentiation of the acinar cells, while the centro-acinar and

ductal cells remained largely unaffected. The relationship of the early functional and structural changes in acinar and ductal pancreatic cells to pancreatic carcinogenesis remains, however, to be seen.

In healthy volunteers Rinderknecht et al (151) have found that numerous lysosomal enzymes are secreted in pure pancreatic juice in a similar manner as digestive enzymes after stimulation with cholecystokinin. This is contradictory to the findings of the same group (156) in patients with cancer of the pancreas, where the activity of lysosomal hydrolases in pancreatic juice - in contrast to that of digestive enzymes - remained within or rose above the normal range. Therefore, the presence of a tumor appeared to have a diametrically opposite effect on the two different groups of enzymes, best illustrated of increasing ratios of lysosomal to digestive enzymes. Thus, concerning changes in lysosomal enzymes during pancreatic carcinogenesis the almost identical findings in the hamster model - at least in this respect - closely resemble human pancreatic cancer development.

In summary, in the Syrian golden hamster changes in pancreatic secretion and enzymes are early and consistent features of the carcinogenic process induced by nitrosamines. If these changes are a consequence of tumorigenesis or if they reflect other cellular events is presently not known.

4.4. Effect of bile deviation on pancreatic carcinogenesis

Most pancreatic cancers are localized to the head of the gland (3). The reason for this has often been discussed, and two hypotheses have gained special interest. The first implies that the predominance to the head is due to the greater mass of ductal cells. Recently, this hypothesis has been questioned by morphometric studies showing that the relative frequency of pancreatic cancers in the head is significantly greater than expected from the amount of ductal epithelium (19). We have tested the other theory that reflux of bile-borne carcinogens and co-carcinogens into the duct of the pancreatic head is responsible for the accumulation of cancers to the pancreatic head. A cholecysto-duodenostomy combined with a short suprapancreatic bile duct resection was found not to influence the carcinogenic action of BHP, administered s.c., for 20 or 24 weeks with respect to frequency of tumor- and cancer-bearing animals, number of tumors and cancers, number of tumors and cancers per tumor- and cancer-bearing animal, and the distribution of tumors and cancers within the gland. Moreover, the histological appearance of the induced lesions (ductal hyperplasia, cystadenomas, papillary adenomas and ductal adenocarcinomas) was uninfluenced by bile deviation.

The exocrine pancreatic cell may be reached by carcinogens via the blood supply, via the pancreatic juice, or by reflux of bile or duo-

denal content into the pancreatic duct(s). Studies of the metabolism of BHP in the hamster (157) have shown that although the main route of excretion of the carcinogens seems to be via the lungs and the kidneys, both HPOP and BOP have been traced in bile and pancreatic juice after administration of BHP i.p. The fact that a high yield of tumors was found in both the treatment and the control group confirms the fitness of the experimental design.

The present study supports and extends the report of Pour & Donnelly (145), who in a small series of 16 evaluable hamsters found no influence of bile deviation on the carcinogenesis of BOP. They have extended their study (Pour, personal communication) by deviation of the pancreatic duct into the sigmoid colon with similar results. In contrast to the results of our study and to those described by Pour & Donnelly, Rückert et al (146) reported a markedly decreased tumor incidence after bili-digestive anastomosis in hamsters given BHP s.c. for 15 weeks. However, like us, Rückert et al found no accumulation of tumors to the head of the pancreas in any group which speaks against the reflux theory. The finding of a decreased tumor frequency after choledocho-jejunostomy in the study of Rückert et al (146) is sooner related to the use of unoperated control animals than to bile deviation, as it has been suggested that sham operation decreases the frequency of induced tumors (158).

Unfortunately, the cause of the accumulation of pancreatic cancers to the head of the gland is still shrouded in mystery. Future efforts should focus on this problem because increased knowledge would probably help to understand mechanisms involved in pancreatic carcinogenesis.

4.5. Influence of gastrontestinal hormones and of oral trypsin inhibitor on pancreatic carcinogenesis (VI, VII, VIII)

In recent years it has come to be recognized that gastrointestinal hormones of the cholecystokinin (CCK) group exert trophic effects on the pancreas (61). It has also been shown (75) that the pancreatic secretion in the rat is controlled by a negative feedback regulation exerted by intraluminal trypsin. There are some evidence that regenerating tissue may have increased susceptibility to carcinogens (159) it has lead to the suggestion that gastrointestinal hormones may influence the development of pancreatic cancer, e.g., by sensitizing the gland to environmental carcinogens. We therefore found it of interest to investigate whether exogenous or endogenous release of hormones of the CCK-group influences induced pancreatic cancer in the Syrian golden hamster.

In the first part of the study we wanted to make sure that a feedback mechanism exerted by intraluminal trypsin exists also in the hamster. In acute experiments on animals prepared with bile-pancreatic and duodenal fistulae we found that bile-pancreatic secretion was stable

throughout the 14 hours of the experiment as regards secreted volumes and protein output. Infusion into the duodenum of bicarbonate or bile did not affect the secretion. However, when bile-pancreatic juice or trypsin was administered intraduodenally, a marked depression of amylase and protein output was found. After addition of trypsin inhibitor - in a dose sufficient to eliminate all trypsin activity - to either of the two infusates the secretion was restored to the initial values.

These results confirm that the pancreatic enzyme secretion in the Syrian golden hamster is controlled by a negative feedback regulation exerted by intraluminal trypsin. The existence of this regulatory system has earlier been demonstrated in the rat (160), the pig (76) and in one patient - a man with a cancer of the papilla of Vater totally obstructing the outlet of pancreatic duct (77). Using endoscopic cannulation Osnes and Hanssen (79) found an inhibition of pancreatic secretion by duodenal infusion of pancreatic juice. However, Dlugosz et al (78) did not find any such influence in their study using a technique with a double balloon in the duodenum. In this study the trypsin activity was altered by infusion of active trypsin and/or trypsin inhibitor into a restricted (18 cm) portion of the duodenum. This part of the intestine was enclosed by two distended balloons. It is reasonable to assume that there were substantial amounts of trypsin within the jejunum distal to the most distal balloon during the experiments. Therefore, the experimental design was not suitable for use in the studies presented. So, the definite solution of the existence of a feedback mechanism in man is probable but not definitely proven.

In a long-term experiment (10 days) repeated subcutaneous injections of CCK caused a significant increase of pancreatic protein and amylase content, as did oral trypsin inhibitor. Also administration of cerulein (2 µg twice daily for 5 days a week) for 18 or 22 weeks caused in another experiment an increase of pancreatic wet weight by about 100 % and pancreatic protein content by 73 %. The findings confirm that both CCK, cerulein and orally administered trypsin inhibitor exert trophic effects on the pancreas of the hamster, and support the findings of Mainz et al (63) who in the rat found that CCK-administration was associated with increased RNA, protein and DNA content and with stimulation of ¹⁴C-thymidine incorporation into DNA.

Against the above background pancreatic carcinogenesis was studied during concomitant administration of CCK, cerulein or oral trypsin inhibitor. The experiment was run in two series of different duration.

First the effect of CCK, secretin, a combination of both hormones, and the effect of oral trypsin inhibitor were studied in hamsters treated with weekly subcutaneous injections of 10 mg BOP per kg bw for 6 or 9 weeks. In the second part the influence of the CCK-analog cerulein was investigated in hamsters given BHP in a dose of 125 mg per kg bw for

18 and 22 weeks, or BHP in a dose of 250 mg per kg bw for 22 weeks.

In the short-term experiment no influence was found by CCK and secretin alone or in combination or by oral trypsin inhibitor on the number of tumors and cancers totally or per animal, the number of tumor- or cancer-bearing animals, the number of tumors or cancers per tumor- or cancer-bearing animal, respectively, or the appearance or pre-neoplastic changes (tubular complexes or ductal proliferations with marked atypia).

In the cerulein-study administration of the carcinogen BHP made 44 % of the animals tumor-bearing after 18 weeks and 73 % after 22 weeks with a dose of 125 mg per kg bw, and 100 % of the animals had pancreatic tumors after 22 weeks with a dose of 250 mg BHP per kg bw. At neither dose and neither time interval did cerulein influence the number of tumor-bearing animals, number of cancer-bearing animals, number of tumors per tumor-bearing animal or cancers per cancer-bearing animal. No morphological differences of the induced lesions were observed between groups of animals given only BHP compared to those given cerulein in addition, and the distribution of tumors was similar in all groups irrespective of the treatment given.

Johnson et al (161) recently reported that CCK protected the hamsters from developing BHP-induced pancreatic carcinomas. These results, which are contrary to the findings of the present study, are irrelevant if effects of a trophic action are to be studied as CCK was given only once a week in a dose which is too low to cause long-standing effects on the pancreas. Townsend et al (162) studied the effect of cerulein and/or secretin on the growth of pancreatic ductal cancer after transplantation of tumor to the cheek pouch of the hamster. They found that the combination of cerulein and secretin increased the wet weight and DNA-content of the tumors, whereas cerulein or secretin alone were without any effects in this respect. It is surprising that cerulein alone - in adequate dose - was without promoting effects.

Using other experimental models regenerating tissue has been shown to be more susceptible to carcinogens. Konishi et al (163) found that the frequency of acinar tumors induced with 4-HAQO was enhanced after partial pancreatectomy - an operation known to induce regeneration of the pancreas. Furthermore, Konishi et al (132, 164) found an increased 4-HAQO-induced tumor incidence in rats fed an ethionine-free diet. Among dietary factors known to release trophic factors from the small intestine trypsin inhibitors have been experimentally studied. We did, however, not find any promoting effect by such diet (raw soya bean flour) on tumor development in our hamster model for ductal carcinoma. On the other hand Morgan et al (165) recorded a potentiation of the action of azaserine on the rat pancreas by raw soya bean flour, but it should be remembered that azaserine causes tumors of acinar appearance (131). Also, Levison et al (166) found increased carcinogenic effects of BHP in rats on a raw soya bean diet. However, they did not state if

the adenocarcinomas induced were of ductal or acinar type. In summary it seems as if a trypsin inhibitor diet does not influence ductal tumors - as in the hamster - but may have a promoting effect on acinar tumors which could be a reflexion of the acinar cell being the target of CCK.

4.6. Prerequisites for estrogen-related treatment in pancreatic cancer (IX, X)

The presence of specific receptors in or on a cell suggests hormonal influence on the metabolism or function of the cell and may further define hormonal dependency on the disease state. In the evaluation of the fitness of the hamster model of induced ductal pancreatic carcinoma for studies on estrogen and estrogen-mediated effects on pancreatic cancer we found that an intravenous injection of polyest-radiolophosphate (PEP) was without influence on unstimulated pancreatic protein and amylase secretion. Further, a single injection of PEP did not change the pancreatic weight, protein- and amylase-content in a ten days experiment, nor did seven monthly injections of PEP change the histological appearance of the pancreas. The dose of PEP used (a single injection of 1 mg/kg bw) was chosen as it is known to induce cancer in other organs (kidney) (167).

Sandberg & Rosenthal (85) found retention of ^3H -estriol after an intravenous injection of this compound in several non-reproductive organs in the dog, e.g. liver and kidney, but most of the radioactivity was retained in the pancreas. Similar results were obtained in the rat, guinea-pig and baboon for estriol as well as for estradiol and diethylstilboestrol. At autoradiography estradiol was found to be localized preponderantly to the acinar cells. After adrenalectomy in male rats, and after adrenalectomy and ovariectomy in female rats Grossman, Bector & Lane (168) found that the acinar cells of the pancreas were degranulated, but a few hours after administration of estradiol there was a complete restoration of zymogen granulation, probably as a result of reduced secretion (169). This is somewhat contrary to the findings of Sandberg & Rosenthal (85) who reported that PEP induced a profound disorganization of the microsomal structure of pancreatic cells in the dog. On the other hand, Bector & Grossman (170) observed significantly reduced amylase secretion within several hours after steroid administration to normal rats. Recently, it was found (171) that somatostatin enhanced binding of estradiol to a cytosolic protein in the rat pancreas. Therefore, one may speculate that exogenous estrogens influence the pancreatic cell only in case of deficiency of endogenous estrogens. Our findings that PEP did not influence pancreatic secretion may thus be due to the presence of normal endogenous estrogen levels.

Estrogen-receptors (ER) have been described in normal exocrine pancreatic tissue of the rat, guinea-pig and baboon (85), in rat pancreatic islets (172), in normal human pancreas (85), in fetal human

pancreas (87), and in human pancreatic carcinoma (87, 173). We found ER in normal hamster pancreas, although in low concentrations, and in even smaller amounts in hamster pancreatic cancer. We also investigated normal pancreas from cow, sheep, pig, guinea-pig, rat and mouse and found ER in the same amounts as in the hamster. ER was not found in liver tissue from any of the hamsters studied. Progesterone-receptors (PgR) were looked for in the same tissues and animals as ER but were not demonstrated. Seven monthly estrogen injections did not change the content of ER in hamster pancreas.

In earlier studies with DMBA-induced pancreatic carcinoma Satake et al (89) found ER in 7 of 16 cancers in the rat. Five ductal pancreatic adenocarcinomas in the Syrian golden hamster, induced with nitrosamines, investigated by Molteni et al (90) were, however, negative with respect to ER. As they chose a value of 6 fmol/mg protein as a dividing line of negative to positive binding, our findings of very low concentrations of ER in normal pancreas and even lower concentrations in pancreatic cancer are consistent with the report of Molteni et al (90). The recent finding of Band et al (171) that somatostatin enhances binding of estradiol to a cytosolic protein, assumed to be ER, in the rat pancreas proposes an interplay of peptides and steroid hormones via ER, and suggests that this effect may come about by a common regulatory mechanism. As functional somatostatin and VIP receptors recently have been detected in a human pancreatic cancer line (Clemente, personal communication) a possible interaction of different kinds of peptides must be considered in further studies on estrogen or estrogen-linked effects in pancreatic cancer.

There are also reports (85) of an estrogen-binding protein (EBP) with a significantly higher binding capacity than ER, and qualitatively different from ER. Using in vitro techniques Poussette and co-workers (86) found EBP to constitute about 4 % of the total protein content in human pancreatic cytosol. Competition experiments indicated that, besides estradiol, the protein also had the same affinity to estrone and estriol, but not to testosterone, progesterone and dexamethasone. We found high concentrations of EBP, about 1 000 times the amount of ER, in cytosols from normal hamster pancreas, and equal amounts in the pancreas from cow, sheep, guinea-pig, rat and mouse. However, EBP was not detected at all in the nitrosamine-induced cancers. We have earlier reported on (172) the finding of high amounts of EBP in the specimen after a total pancreatectomy for a well differentiated ductal carcinoma in man, but no EBP was found in four other investigated human pancreatic cancers. This proposes differences in the content of ER and EBP between human ductal carcinoma and the induced ductal carcinoma in the Syrian golden hamster and seems to imply that this otherwise useful model has got shortcomings in studies on estrogen effects on pancreatic cancer.

In the studies of estramustine, a nor-nitrogen mustard derivative of estradiol we confirmed the presence of EBP in the pancreatic cytosol

from normal hamster. In tumor-free pancreatic tissue from BOP-treated hamsters EBP was measured in an amount corresponding to 10 % of the values found in normal animals. In induced pancreatic tumor no EBP was found at all. As estramustine is a derivative of estradiol we investigated if binding of estramustine to EBP could be responsible for uptake of the compound. However, competition studies with labeled estradiol and unlabeled estramustine showed that estramustine was only very weakly bound to EBP. As the distribution studies showed that estramustine was taken up in pancreatic tumors, although to a lesser degree than in normal pancreatic tissue, this together with the finding of lack of EBP in cancer tissue strongly indicated that the uptake of estramustine in cancerous as well as in normal pancreatic tissue was not caused by binding between estramustine and EBP. Instead the pancreatic uptake of estramustine appears to be attributed to the binding of the compound to a cytosolic protein which is different from EBP. In contrast to EBP this protein was detected in cancerous as well as noncancerous pancreas of the hamster. Thus, the present results suggest that estramustine would be preferable to compounds which bind to EBP, such as estrogens or antiestrogens, in the treatment of pancreatic cancer.

Distribution studies of estramustine in the normal hamster showed that both the estrogen and the mustard part of the molecule were retained in the pancreas to a higher degree than in plasma and muscle. The concentration, as examined by the double-labeling, were initially equal, indicating that the parent estramustine is retained. Later, however the concentration of the mustard part was higher, suggesting that estramustine at least partly is hydrolyzed in the hamster pancreas. When double-labeled estramustine was given to BOP-treated animals the concentrations of radioactivity in different organs were about the same as in corresponding organs in the experiments with untreated animals, which indicates that the distribution of estramustine in the Syrian golden hamster is uninfluenced by the carcinogen administration per se.

In summary the investigations of the actions of estrogen and the function of ER have given rise to several important issues. Firstly, ER and EBP have been shown to be present in normal but not cancerous hamster pancreas, which implies that there is a qualitative difference between the hamster model and the human cancer. Secondly, it seems as if administration of estrogen to normal pancreas does not influence its morphology or function. Thirdly, lack of EBP is an indicator of malignant pancreatic disease, and fourthly, there is a cytosolic protein binding estrogens and to a higher degree estramustine different from EBP in normal as well as cancerous pancreas. As estramustine, a cytotoxic drug, is retained in the pancreatic tissue, normal as well as cancerous, selective treatment of pancreatic cancers might be possible. This warrants that further studies in this field should be given a high priority.

5. POSSIBLE PATHWAYS FOR FUTURE WORK

For patients with gastrointestinal malignancies the only hope for cure is surgery. In carcinoma of the stomach or the colon the chance for the patient to be restored to health is substantial, but in pancreatic cancer still remains close to zero. The results of the clinical part of the present thesis underline the ultimate dismal prognosis for patients with pancreatic cancer - only 4 out of 85 patients in this selected group of patients survived more than 5 years. However, the clinical findings create guidelines for improved management of the patients. It seems e.g. highly important to adopt a selective attitude. Only patients with tumors without known growth outside the pancreas, adequately investigated preoperatively and considered able to withstand major surgery should be selected. The operation should be done in a hospital with facilities for peroperative histological examinations and with experiences of this particular type of operative and postoperative treatment. Using these principles of management a reduction in hospital mortality is to be expected.

In an attempt to improve long-term prognosis two different ways of action can be chosen. In most cases an extensive surgical procedure is not curative. Hitherto, additional treatment such as chemotherapy and radiotherapy, has been used mainly in patients with inextirpable tumors. I find it, however, more logical to concentrate these kinds of treatment of adjunctive measures on patients with potentially curative disease. Also, more effective adjunctive treatments must be looked for and may be hormonal or hormone-linked treatment modalities can offer some benefit.

Moreover, there is a strong need to identify the patients with pancreatic cancer at an earlier stage; the present thesis emphasizes the importance of the stage of the disease as prognostic factor. Presently there are no sensitive and specific tumor markers for pancreatic cancer available to be used for screening of risk-groups or patients with vague symptoms. The changes in lysosomal enzymes found during pancreatic carcinogenesis might offer a possibility for development of tools for early diagnosis; the differences between normal and cancerous tissue with respect to estrogen- and estramustine-binding proteins may be another.

The incidence of pancreatic cancer in Sweden seems to display an altered time-trend, i.e. no further increase but rather a decreasing incidence seems to be under way. Careful analysis of possible causes of this changing pattern may throw a new light on the etiology of the disease. More over, the differences in prognosis found between men and women are to be further explored both epidemiologically and in experimental models.

The reason for predominance of the cancer to the head of the pancreas is still not known. If this problem could be solved research on the

pathogenesis of the pancreatic cancer could possibly be made more precise, and I look upon this as a high priority research field.

Earlier experimental work has shown that the composition of the diet influences the relative proportions of enzymes within the pancreas and pancreatic juice and the synthesis of pancreatic enzymes. Our studies have shown that a feed-back regulation of pancreatic enzyme secretion exerted by intraluminal trypsin exists in Syrian golden hamster, as has been earlier shown in rat and pig. Protein and dietary fiber have been proposed to interfere with this regulatory mechanism. This finding together with the known trophic effects on the pancreas of certain gastrointestinal hormones shown i.e. in this study, provide directions for further direct or indirect studies on the association between dietary components and pancreatic carcinogenesis, although gastrointestinal hormones did not influence carcinogenesis in our study. However, I find it important that the role of diet is further studied not only as a regulator of physiological processes within the pancreas, but also as a promotor of pathophysiological effects and may be even as a direct carcinogen or co-carcinogen factor.

The findings of estrogen-receptors, estrogen-binding protein and estramustine-binding protein deserve attention in future work on pancreatic cancer. On-going research shows that there might be an interplay between the steroid and the gastrointestinal hormones in the action on the normal pancreas. The significance of this remains to be elucidated, as well as its possible role in the pathogenesis of pancreatic cancer. Further, the relationship between the estrogen-receptors and the estrogen-binding protein is not known. If estrogen has a potentially trophic influence on the pancreas, or if lack of estrogenic action on the gland inhibits the protein synthesis manipulations of both the ER and EBP might be necessary for the possible influence on pancreatic carcinogenesis. On the other hand, perhaps the estrogens bound to ER and EBP have different effects and may be manipulated in different ways, e.g., it is known that tamoxifen blocks the action of ER but has a low affinity to EBP. Moreover, the findings of high levels of hormones converting testosterone to estrogen (i.e. aromatase and 5- α -reductase) in pancreatic cancer tissue combined with low levels of circulating testosterone raises the question if the antiandrogen cyproteroneacetat and/or antiadrenal agent aminogluthemide could be used as an adjunctive treatment in pancreatic carcinoma. Also, our findings of qualitative differences between EBP and estramustine-binding protein, EmBP, might be useful to selectively get EmBP-bound drugs into the pancreatic cancer. Hitherto, we have only investigated estramustine in this respect, but most probably derivatives of estramustine - and perhaps other drugs - can be bound to EmBP; drugs that might have beneficial effects compared to estramustine. If deeper knowledge in the biochemistry and physiology of ER, EBP and EmBP is achieved it is possible that hormone-linked approaches can be used for diagnostic as well as for therapeutic purposes.

6. CONCLUSIONS

From the results of the thesis the following conclusions are drawn:

- 1/ Factors influencing hospital mortality after total pancreatectomy for pancreatic cancer should be possible to control, whereas factors related to long-term prognosis presently can not be influenced.
- 2/ The delivery of the model for experimental pancreatic cancer induced by nitrosamines in the Syrian golden hamster is possible to modulate by changing carcinogen dose and induction time, and, provided adequate control groups are used, the model is suitable as well as reliable for studies of factors thought to be modulators of tumor development.
- 3/ In the Syrian golden hamster changes in pancreatic secretion and enzymes are early and consistent features of the carcinogenic process induced by nitrosamines. The level of lysosomal enzymes, in contrast to that of digestive enzymes, increases during carcinogenesis.
- 4/ In the model used bile-deviation from the pancreas does not influence pancreatic carcinogenesis, contradicting that reflux of bile-borne carcinogens and co-carcinogens into the pancreatic duct of the pancreatic head is of pathogenetic importance.
- 5/ Pancreatic enzyme secretion in the Syrian golden hamster is controlled by a negative feedback regulation exerted by intraluminal trypsin.
- 6/ Parenteral administration of cholecystokinin and cerulein, and orally administered trypsin inhibitor exert, in contrast to secretin, trophic effects on the pancreas in the Syrian golden hamster.
- 7/ Parenteral administration of cholecystokinin and secretin, alone or in combination, or cerulein and orally administered trypsin inhibitor do not influence pancreatic carcinogenesis in the Syrian golden hamster.
- 8/ Estradiol is without influence on basal pancreatic protein and amylase secretion. Neither does estradiol change pancreatic weight, protein or amylase content in the Syrian golden hamster.
- 9/ Estrogen-receptors are found in low concentrations in normal hamster pancreas and in even smaller amounts in the induced pancreatic cancers. Progesteron-receptors are undetectable in normal as well as in cancerous pancreas of the hamster.

- 10/ Estradiol-binding protein is found in high amounts in healthy pancreas in the Syrian golden hamster, cow, sheep, pig, guinea-pig, rat and mouse, but not in the induced pancreatic cancers in the hamster.
- 11/ Estramustine, a nornitrogen mustard derivative of estradiol, is taken up in the pancreas of normal Syrian golden hamster, and, although to a lower degree, in induced pancreatic cancer.
- 12/ Estramustine uptake in the hamster pancreas is attributed to the binding to a cytosolic protein, present in normal pancreas as well as in pancreatic tumors, different from the estradiol binding protein.

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FACTORS INFLUENCING SURVIVAL AFTER TOTAL
PANCREATECTOMY IN PATIENTS WITH PANCREATIC CANCER

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Abstract

A retrospective analysis of factors influencing short-term and long-term survival after total pancreatectomy for pancreatic cancer was done in 86 patients. Among the 41 factors studied hospital mortality was significantly affected by age over 70 years, preoperative diabetes, pain as presenting symptom, S-bilirubin, preoperative bile drainage, prophylactic antibiotic treatment, stage of the tumor and experience of the surgeon. The only factors which had a statistically significant influence on long-term survival were stage of the tumor and sex of the patient.

It is concluded that improvement of long-term survival can mainly be achieved by earlier identification and removal of the tumors and by introduction of more efficient adjuvant therapy. Whereas these goals probably will require a long time to be reached, the majority of factors associated with worsening of hospital mortality may be avoided by a strict selection of the patient, the tumor and the surgeon.

Key words: Hospital mortality, long-term survival,
pancreatic cancer, prognostic factors,
total pancreatectomy

The only chance for cure in pancreatic cancer is a resective procedure. Irrespective of the type of resection used, reported results continue to be dismal both regarding hospital mortality, morbidity and long-term survival (1,2,3). Factors influencing the outcome of radical surgery in pancreatic cancer may be related to patient selection, stage of the tumor, surgical technique and postoperative management. As to surgical techniques and experience of the surgeon, Warren (4) and Howard (5) have reported long personal series without any operative deaths. Gilsdorf and Spanos (6) have analysed factors affecting morbidity and mortality in pancreaticoduodenectomy (Whipple procedure) for cancer of the pancreas and bile ducts. For more than 20 years at our hospital, total pancreatectomy has been the routine method when radical surgery has been attempted in patients with pancreatic cancer. During the years the criteria for selection of the patients have varied, the preoperative work-up and postoperative management of the patients have changed and a great number of surgeons have been involved in the operations. We therefore found our series suitable for an analysis of different factors which may be involved in the outcome and prognosis of patients subjected to total pancreatectomy for pancreatic cancer.

Patients and methods

The material comprises 90 patients with adenocarcinoma of the pancreas having total pancreatectomy between 1959 and 1982. Another 27 patients who had the same procedure for cancer of the papilla of Vater, bile ducts or duodenum during the same period were not included. During the 23-years period, a total of 641 patients with carcinoma of the exocrine pancreas were treated at the hospital giving a resectability rate of 14% (90/641). No other types of surgical procedures were done for attempted cure in pancreatic cancer during this period, except in one patient whose operation was a Whipple procedure. The records of four patients were inadequate. Therefore data from 86 of the patients were finally available for analyses. Forty-one different variables - listed in table I - were evaluated in this retrospective study.

In the majority of patients the operation was done as previously described (7) with removal en bloc of the great omentum, pancreas, duodenum, common bile duct, gall-bladder, spleen and at least 50% of the stomach.

The patients were staged according to a modification of the classification described by Hermreck et al (8): stage_I means that the tumor is localized within the pancreatic capsule, stage_II: that the tumor invades the duodenum, stage_III: that there is an invasion of the superior mesenteric or portal vessels or regional lymph nodes and stage_IV: that

Table I. Factors evaluated with regard to survival after total pancreatectomy in 86 patients with pancreatic cancer.

<u>Factors related to the patient (host)</u>	<u>Factors related to the treatment (management)</u>
Age	Primary operation for the disease
Sex	Preoperative bile drainage
Occupation	Preoperative histological or cytological verification of the diagnosis
Smoking habits	Preoperative maximal S-bilirubin, S-ALP
Alcohol consumption	S-bilirubin, S-ALP immediately preoperatively
Place of dwelling	Preoperative S-albumin
Heridity for exocrine pancreatic disease	Bacterial growth in preoperatively externally drained bile
Heridity for endocrine pancreatic disease	Prophylactic antibiotic therapy
Presenting symptoms (jaundice, weight loss, pain)	Prophylactic anti-trombose regimen
Preoperative diabetes	Year of operation
Preoperative cardiac disease	Experience of the surgeon
Preoperative arterial hypertension	Duration of operation (anesthesia)
Other preoperative diseases of importance	Number of blood transfusions preoperatively
Other longterm preoperative medication	Number of blood transfusions peroperatively
Previous abdominal surgery for other diseases	Number of blood transfusions postoperatively
<u>Factors related to the tumor</u>	Peroperative complications (difficulties)
Location	Postoperative complications
Size	Time in Intensive Care Unit
Histological grade	Duration of postoperative hospital stay
Stage (according to Hemreck)	Postoperative diabetes
Multicentricity	Postoperative maldigestion

there is distant spread.

Generally hospital mortality is defined as death within the first 30 days after the surgical procedure. In this series, however, seven (28%) of the patients who succumbed in connection with the pancreatectomy died after the first postoperative month. Therefore patients who died without leaving the hospital after the operation were considered as hospital deaths. With this definition hospital mortality was 29% (25/86) as compared to 21% (18/86) if the 30-days mortality was calculated.

The statistical analyses were done by using Chi-square test with Yates correction when comparing two factors, Chi-square test for trends in proportions when comparing three factors and generalized Wilcoxon test when comparing factors affecting long-term survival.

Results

Of the 41 variables analysed, those with a statistically significant correlation to hospital mortality or long-term survival will be reported on. Thus, other variables (table I) - not mentioned under "Results" - were found not to be associated with the outcome of the disease.

Host factors affecting hospital mortality

Among the patients suffering hospital mortality 76% (19/25) were men which is as much as 40% (19/47) of all male patients. The corresponding figure in female patients was 18% (6/34) but no statistically significant difference between the sexes was found in this respect. The mean age $\pm$ SD was 62.9 ± 7.0 in the hospital mortality-group and 60.5 ± 9.4 in those who survived the operation. Likewise there was no difference in age between men suffering hospital mortality as compared to women. When, however, analysing separately patients 70 years of age or more it was found that 58% (7/12) did not get over the operation compared to 24% (18/69) for patients younger than 70 ($p < 0.05$).

Previous or concomittant diseases did not influence hospital mortality except for the presence of preoperative diabetes. As shown in figure 1, 28% (7/25) of the patients who suffered hospital mortality were diabetics as compared to 8% (5/61) among those who survived the operation ($p < 0.05$). Abdominal and/or back pain was the primary symptom in 44% (11/25) of patients of the hospital mortality group as compared to 18% (11/61) in those surviving the operation ($p < 0.05$).

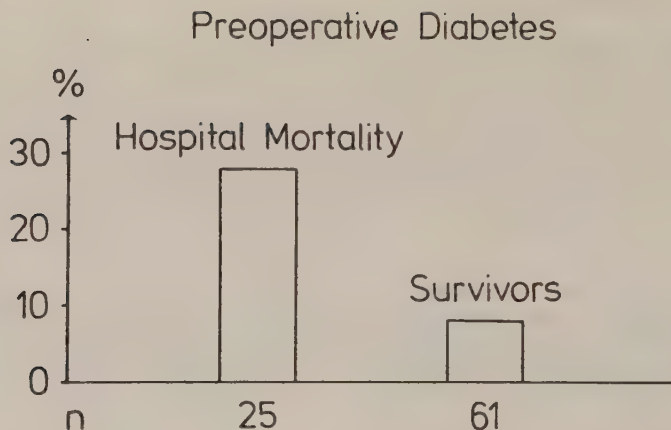


Fig 1. The frequency of preoperative diabetes mellitus in 86 patients succumbing in connection with (n = 25) or surviving (n = 61) a total pancreatectomy for pancreatic cancer.

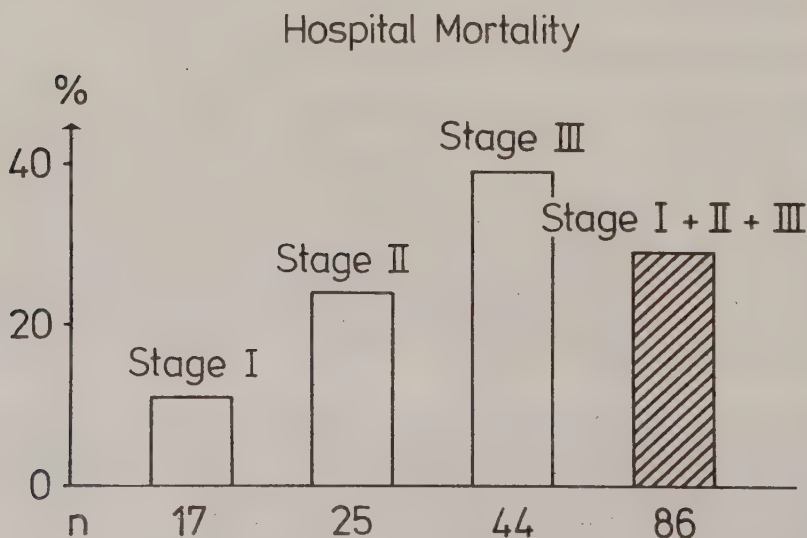


Fig 2. Hospital mortality after total pancreatectomy in 86 patients with pancreatic cancer related to stage of the tumor.

Characteristics of the tumor affecting hospital mortality

The estimated size of the tumor was not found to influence hospital mortality. It should, however, be pointed out that in these selected cases most lesions were between 2 and 3 cm in diameter and no tumor was bigger than 5 cm.

Figure 2 shows that hospital mortality was 12% in stage I, 24% in stage II and 39% in stage III ($p < 0.05$). The grade of histological differentiation of the tumor did not influence hospital mortality.

Factors related to management and surgery affecting hospital mortality

Whether a primary laparotomy had been done or not was of no importance for hospital mortality. A statistically significant difference ($p < 0.05$) was found between patients with and without preoperative bile drainage (operative or non-operative percutaneous procedures) as shown in figure 3.

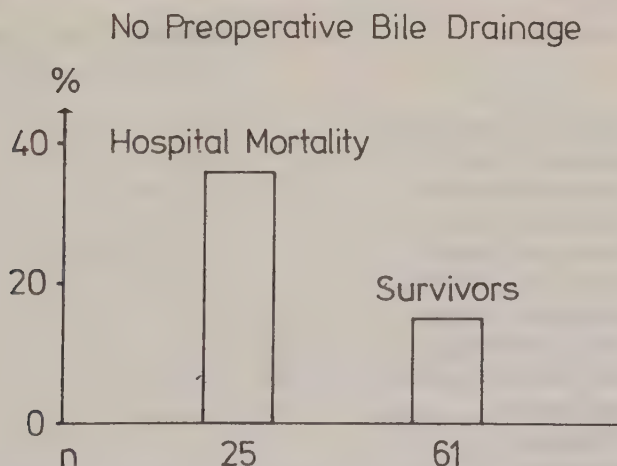


Fig 3. Hospital mortality in patients with and without a preoperative bile drainage prior to total pancreatectomy for pancreatic cancer.

Similarly, 72% (18/25) of patients suffering hospital mortality had increased S-bilirubin ($>$ double the upper limit of normals) the day of operation compared to 36% (22/61) among those surviving the operation ($p < 0.05$).

Prophylactic antibiotic treatment was given to 32% (8/25) of

patients belonging to the hospital mortality group and 85% (52/61) of the survivors ($p < 0.05$). Neither operation time nor number of blood transfusions given during the operation was correlated to hospital mortality. Although there was a tendency for hospital mortality to decrease with time (year of operation) no statistically significant difference could be achieved. The experience of the surgeon was a factor difficult to evaluate in detail as more than one experienced surgeon rather often took part in the operation. It is, however, evident that surgeons who in this series did 10 or more operations experienced a hospital mortality of 10%, which is significantly lower than the corresponding over-all figures.

The complications leading to hospital mortality is shown in table II in relation to the duration of survival. As can be seen 8/25 patients died within the first week.

Table II. Complications leading to hospital mortality in relation to the time of death (n = 25).

Cause of death	<u>Postoperative day of death</u> (one figure = one patient)
Anastomosis insufficiency	18, 21, 38, 40
Abdominal abscess	22, 23
Intraabdominal bleeding	0, 4, 12, 17
Renal insufficiency	5, 6, 15
Cardiac insufficiency	1, 45, 47, 59, 63
Respiratory insufficiency	9
Haemorrhagic gastritis	45
Gastric perforation	13
Small intestinal gangrene	11
Liver ischemia	3
Gas gangrene	1
Hypoglycemia	4

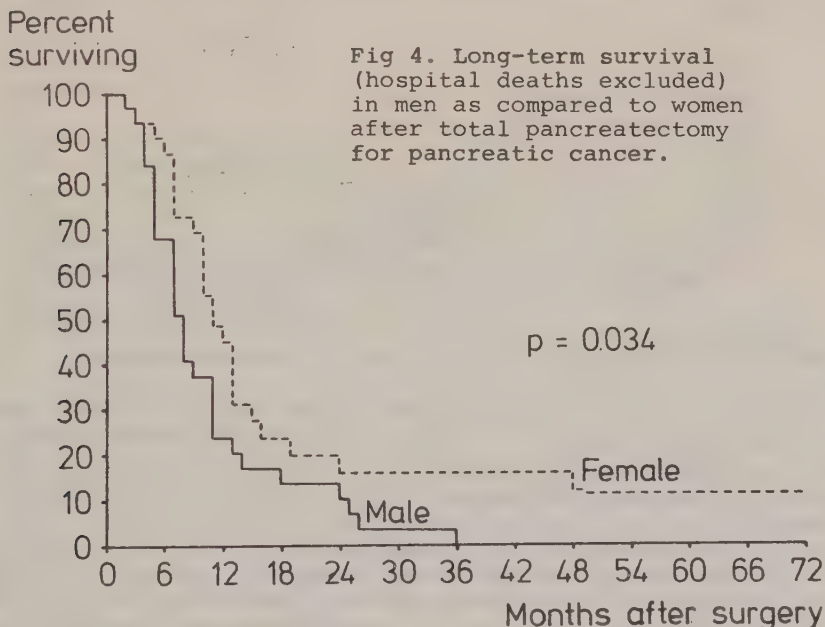
It is interesting to note that one patient who did not leave the hospital alive died in connection with an attack of severe hypoglycemia. It should, however, be pointed out that this death occurred 19 years ago.

Host factors affecting long-term survival

When calculating factors related to long-term survival patients suffering hospital mortality were excluded. The age of the patient at operation did not influence the prognosis of the

disease. It is of interest to note that the five oldest patients surviving the operation and who were 71, 73, 73, 74 and 78 years of age lived for 19, 13, 26, 11 and 9 months, respectively, averaging 16 months which is to be compared to a mean survival of 14 months in patients younger than 70 years of age.

As shown in figure 4 females surviving the operation seemed to have somewhat better prognosis ($p < 0.05$) than male patients. Whereas the one-year survival was 25% for men and 46% for women the difference, however, had diminished to 10% and 16%, respectively, already after two years.

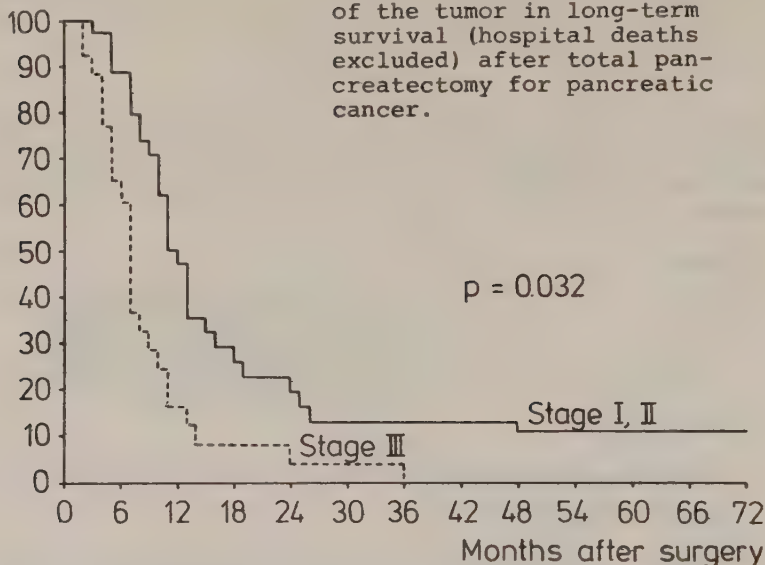


Characteristics of the tumor affecting long-term survival

Tumor size was found not to be correlated to long-term survival possibly reflecting the selection of relatively small tumors for surgery. The stage of the tumor, however, seemed to be an important prognostic factor ($p < 0.05$).

In stage I, II and III the one-year survival was 56%, 36% and 13% and the two-year survival 34%, 11% and 4%, respectively. However, there was no statistically significant difference between stage I and II. Therefore these two stages are put together in figure 5. The histological grading of the tumor

Percent
surviving



was found not to be correlated with long-term survival.

Among the 61 patients surviving the operation 54 (89%) died from recurrence of the disease. Seven patients died from other causes between two months and seventeen years after the operation. In three of them a recurrence was found at autopsy. The one, who died after two months, had a lethal attack of hypoglycemia (in 1961, five weeks after she had left the hospital in good condition). The patient who lived for 17 years had no signs of recurrence at autopsy but death was a result of a progressive uremia (chronic pyelonephritis). The two other patients without signs of recurrence both died after three months. Overall the five-year survival in the present series was 5% (4/85). As all four 5-years-survivors belonged to stage I and II the corresponding figure for these two stages was 10% (4/42). Three of the patients who lived five years or more died after 6, 6 and 17 years, respectively, while one patient is still alive after 7 years.

Factors related to management and surgery affecting long-term survival

The year of operation or experience of the surgeon were without influence on long-term survival. As mentioned above, two patients (2/86) succumbed in connection with a hypoglycemic

attack, one while still in hospital and the other a few weeks after the patient had returned home. In none of the 61 patients surviving the operation was there any reason to anticipate that death was a consequence of maldigestion due to exocrine pancreatic insufficiency.

Discussion

In the present study an attempt has been made to analyse factors affecting hospital mortality and long-term survival in patients operated on with total pancreatectomy for pancreatic cancer. We have done this being well aware of the problem of covariation of several factors. We still believe, however, that analyses of this type are valuable in creating recommendations and guide-lines for the selection of patients who may benefit from a certain therapeutic procedure or types of procedures.

Put against the backdrop of the gloomy long-term survival in "radically treated" pancreatic cancer, hospital mortality should be reasonably low in order to warrant the use of attempted curative procedures in this disease. The key-word for the efforts to reduce hospital mortality after pancreatectomy seems to be selection; selection of the patient, selection of the tumor and selection of the surgeon.

The factors that appeared to influence hospital mortality in the present study were age of 70 years or more, preoperative diabetes, pain as presenting symptom, S-bilirubin, preoperative bile drainage, prophylactic antibiotic treatment, stage of the tumor and the experience of the surgeon.

Although the number of patients 70 years of age or more was small ($n = 12$) the proportion of operative deaths in this group (58%) was significantly higher than in the group of patients younger than 70 years (24%). These data, however, contradict those of Gilsdorf and Spanos (6) who reported only one operative death among eight patients over 75 years of age who had a pancreatico-duodenectomy (Whipple). We believe that the findings of the present study suggest that a highly strict selection of patients of old age for pancreatectomy is advisable.

If pain was an initiating symptom the risk of surgical death was increased. This observation was not found to be a reflection of the stage of the tumor in this series where all patients had their lesions confined to the head of the pancreas.

The stage of the tumor as evaluated by a modification of the Hermreck classification (8) was obviously a significant factor. Considering these observations - and the association of stage of the tumor and long-term survival (vide infra) - it

is a matter of course that there is a need for an internationally accepted clinically useful classification and staging of pancreatic cancer, as emphasized by Pollard et al (9).

A statistically higher hospital mortality did occur in patients without preoperative bile drainage. Similar findings were made by e.g. Gilsdorf and Spanos (6), Braach and Gray (10) and Maki et al (11) who, like us, reported on retrospective studies. On the other hand Norlander et al (12) did not find any influence on hospital mortality of preoperative bile drainage in a group of patients operated on with different palliative and attempted curative procedures. Similarly data from prospective studies do not speak in favour of a reducing effect on hospital mortality by preoperative bile drainage (13,14). It should, however, be stressed that these latter studies still contain a small number of patients and also comprise patients operated on with different kinds of palliative and curative operations. Therefore, the old controversy of the value of preoperative relief of biliary stasis in patients with pancreatic cancer to be operated on with curative procedures must await further investigations.

Prophylactic antibiotic therapy has been shown in prospective studies to be of value in e.g. surgery of the colon (15). In this retrospective investigation there was a lower hospital mortality in the group of patients given prophylactic antibiotics. This shows the need for controlled prospective studies on the value of prophylactic antibiotic therapy in radical pancreatic surgery.

Our data suggest that the experience of the surgeon affects the chances for the patient to survive the operation. This opinion has also been put forward by other authors (3,6) and is further supported by the long personal series without hospital mortality of Warren (4) and Howard (5). In the present study the operations were done by 22 surgeons during a 23-year period. The results emphasize that there is no judicious reason to omit a strict selection of the surgeon or surgical unit. The only report of dissenting opinion known to us is that of Bloom and Steer (16) who recommended pancreatectomy to be performed at any hospital irrespective of the frequency of pancreatic cancer and of the surgeon's experience in pancreatic surger.

Long-term survival was found to be influenced by sex of the patient and stage of the tumor. As death was associated with hypoglycemia in two of the 86 patients this complication should also be taken into consideration. It is, however, important to stress that the two deaths due to hypoglycemia occurred 19 and 21 years ago, respectively. Today the management of the postoperative diabetes seems to be safer.

In the present study females had somewhat more favourable long-term survival than men. Similar observations were made by van Heerden et al (17) after total pancreatectomy for ductal adenocarcinoma. In both series an association was found between female sex and less advanced stage of the tumor. In their study van Heerden et al (17) found that multicentricity of the tumor had a negative influence on survival which was statistically significant at least after one year. Similar findings were not made in the present study. It seems, however, to be especially difficult to analyse multicentricity retrospectively.

The results show that the factors related to hospital mortality are such that they should be possible to control whereas the factors related to long-term survival seem to be more difficult to affect. Because of the dismal long-term results of pancreatectomy, hospital mortality should be less than 5-10% in order to make this kind of treatment meaningful. This is a goal which is possible to achieve if selection of the patient, tumor and surgeon is done after considering the causes of failures done in the past. This would require a centralization of the patients who are candidates for attempted radical surgery to certain speciality centers in order to carry out a standardization of the preoperative and intra-operative evaluation of resectability and to enable a uniform system of surgical techniques, postoperative care and follow-up. On the other hand improvement of long-term survival still must await an earlier identification and diagnosis of the disease and development of better adjuvant treatments.

The question which type of resection should be used in attempted cure of pancreatic cancer was not a topic of this paper. Well aware that total pancreatectomy always causes diabetes and malabsorption and that a weak point of the Whipple procedure is the pancreatico-jejunal anastomosis we still think that most findings of the present study on total pancreatectomy is applicable also on the Whipple procedures.

Acknowledgements

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SEQUENTIAL STUDIES ON THE DEVELOPMENT OF NITROSAMINE-
INDUCED PANCREATIC CARCINOMA IN THE SYRIAN GOLDEN HAMSTER

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Running title: Development of nitrosamine-induced pancreatic
carcinoma

Abstract

An attempt was done to define the importance of accumulated carcinogen dose and of induction time on the development of pancreatic tumors after administration of N-nitrosobis(2-hydroxypropyl)amine (BHP) to Syrian golden hamsters. The development and progression of ductal hyperplasia, number of tumor-bearing animals, number of cancer-bearing animals, number of tumors per tumor-bearing animal, the grade of histological differentiation was found to be related to the accumulated dose of the carcinogen given as was survival time. The duration of the period between the carcinogen administration and examination was another factor of importance for the same changes except for the grade of histological differentiation of cancers and for survival time. Once ductal hyperplasia and tumor development had started there were no signs of regression - but sooner progression - even after cessation of carcinogen administration.

It is concluded that the development (progression) of induced pancreatic cancer in the Syrian golden hamster is influenced by the accumulated carcinogen (BHP) dose and duration of the experiment. The results emphasize the need for a strict standardization of the experimental design as well as for the histological evaluation when animal models are used for studies of the effect of factors or procedures with possible influence on the rate of tumor development.

Key-words: pancreatic carcinogenesis; N-nitrosobis(2-hydroxypropyl)amine (BHP); Syrian golden hamster.

The model for induction of ductal pancreatic carcinoma in the Syrian golden hamster by means of certain nitrosamine derivatives has been frequently used since it was described by Pour et al (6) in 1974. As tumor frequency using this model seems to differ markedly in different reports, also when identical carcinogens and dosages are used (3,7,8) one purpose of this study was to define factors of importance for the rate of sequential tumor development (1,9). This was regarded especially important before starting studies aiming at promotion or retardation of tumor development. As little is known about the ability of pre-cancerous or cancerous lesions to regress, another aim was to investigate if nitrosamine-induced changes in the hamster pancreas at any time after the period of carcinogen administration showed any signs of regression with regard to frequency and morphology.

MATERIAL AND METHODS

One hundred and sixty-four 8 weeks old Syrian golden hamsters from own breeding were used. Pairs of animals were housed by sex in plastic cages. Equal numbers of both sexes were used in the different groups. The animals were fed a standard pellet diet and had free access to tap water. N-nitrosobis(2-hydroxypropyl)amine (BHP) was purchased from Ash Stevens Inc., Detroit, USA. Each animal was given 125 mg BHP/kg body weight dissolved in 0.9% saline by weekly subcutaneous injections. The design of the study is demonstrated in fig. 1. Hamsters belonging to group A were

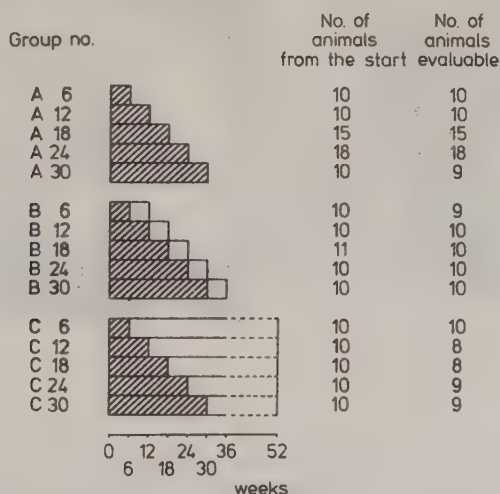


Fig. 1. Design of the study. ▨ = period of carcinogen administration. □ = carcinogen-free period. In group A the animals were killed in the end of the week they got the last carcinogen injection, in group B the animals were treated six weeks after the week they got the last carcinogen injection and in group C 52 weeks after the start of the experiment. The figures 6, 12, 18, 24 and 30 in the left column represent number of weekly carcinogen injections.

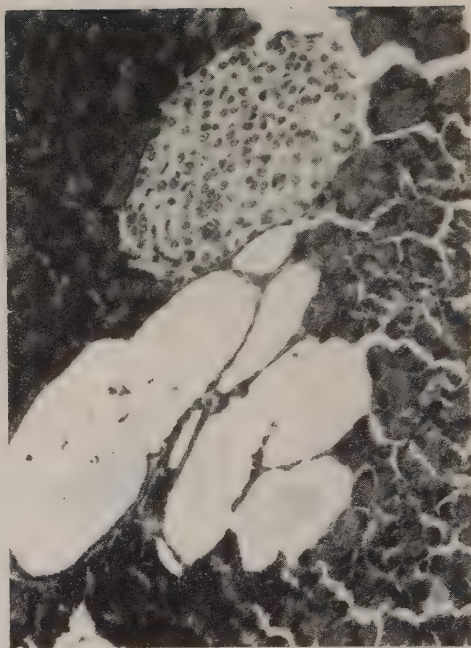


Fig 2. A. Degree of proliferation. Slight proliferation of ducts and ductules, i.e. 1-2 focuses of proliferation per low-power field (x 25). Magnification: left x 20, right x 250.

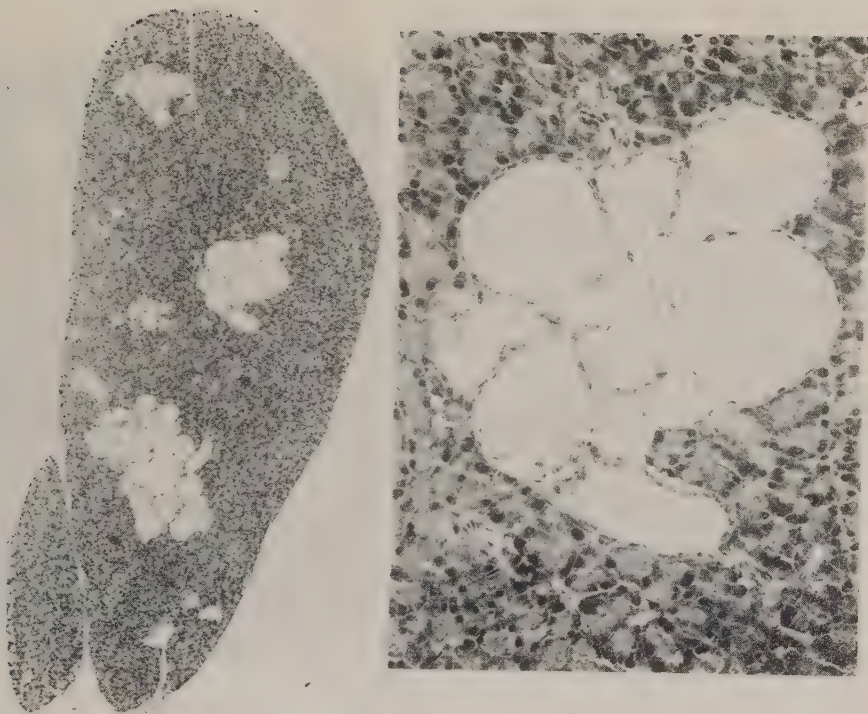


Fig 2. B. Degree of proliferation. Moderate proliferation, i.e. 3-4 foci of proliferation per low-power field. Magnification: left x 40, right x 250.

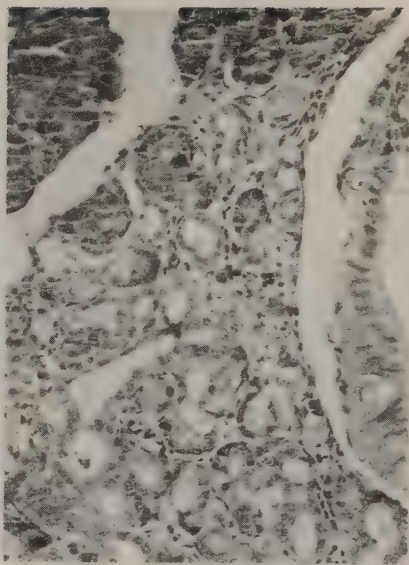
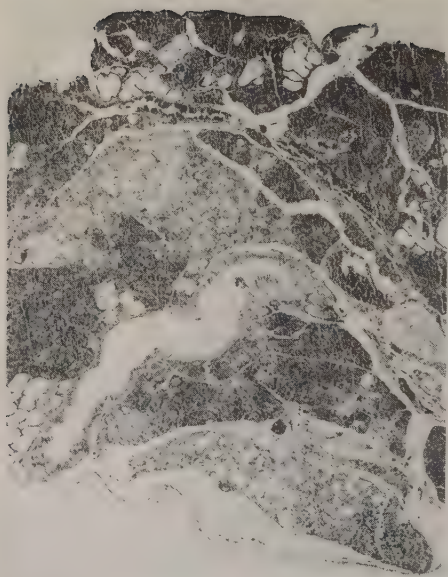


Fig 2. C. Degree of proliferation. Severe proliferation, i.e. more than 4 focuses of proliferation per low-power field. Magnification: left x 47, right x 250.

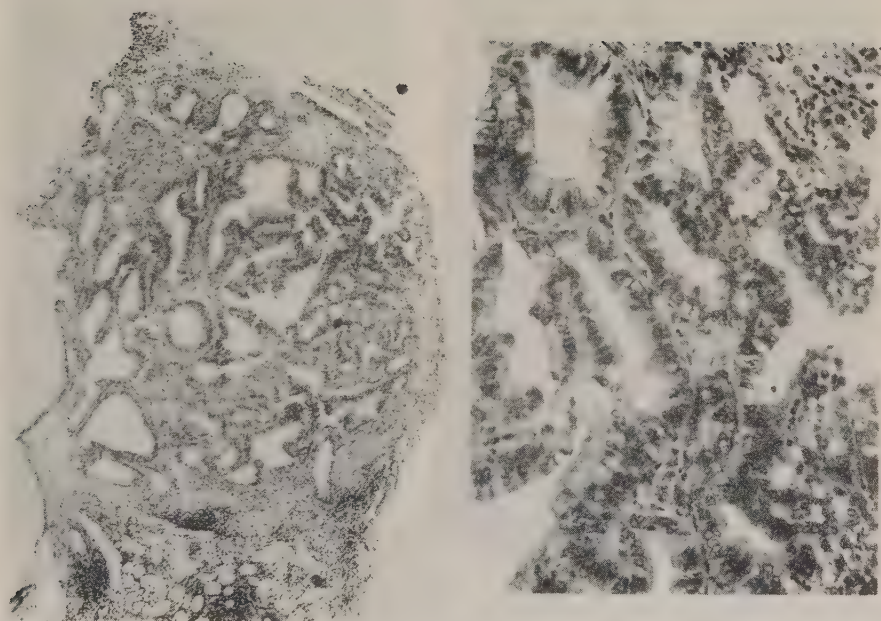


Fig 3. A. Degree of differentiation of carcinomas. Highly differentiated adenocarcinoma. Magnification: left x 55, right x 250.

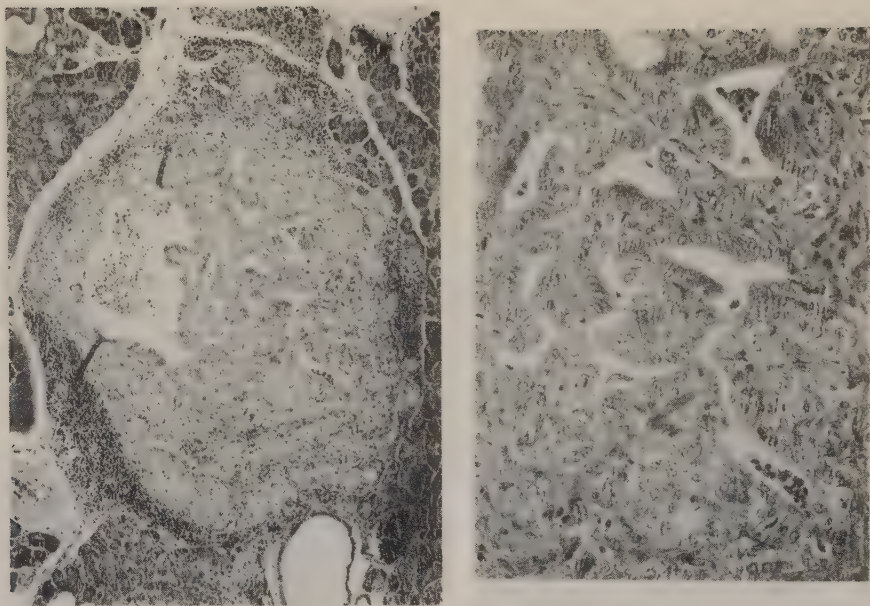


Fig 3. B. Degree of differentiation of carcinomas. Moderately differentiated adenocarcinoma. Magnification: left x 88, right x 250.

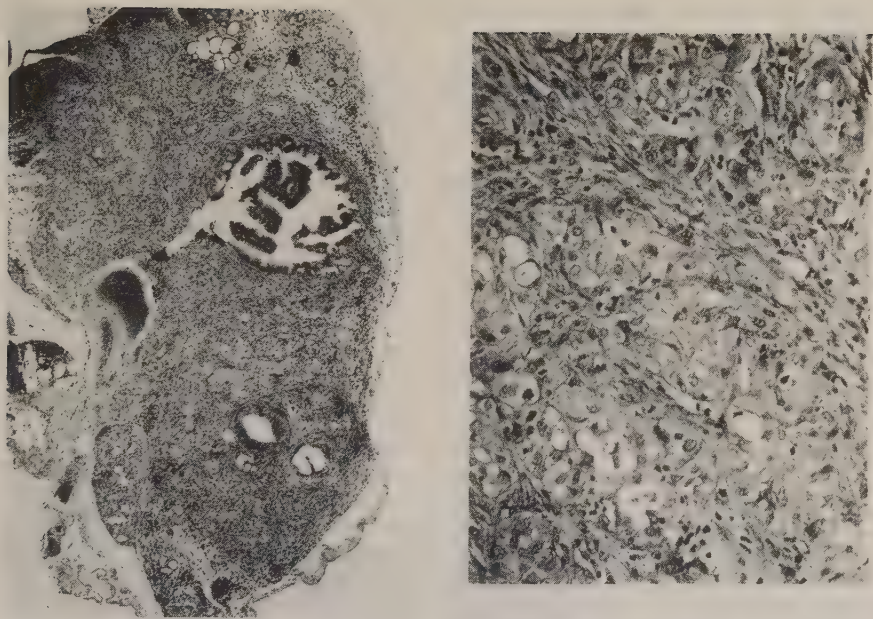


Fig 3. C. Degree of differentiation of carcinomas. Poorly differentiated adenocarcinoma. Magnification: left x 39, right x 250.

sacrificed at the end of the week they got their last carcinogen injection, hamsters of group B were sacrificed after six carcinogen-free weeks and those of group C were allowed to live for 52 weeks including the weeks of carcinogen administration. Animals that succumbed before the end of the experimental period were also included in the study if their bodies were free from autolysis. Each of groups A, B and C were divided into five subgroups given weekly carcinogen injections for 6, 12, 18, 24 or 30 weeks. They were designated as subgroups 6, 12, 18, 24 and 30, respectively. As further seen in figure 1, one animal from group A and six from group C were not amenable for histological examination due to autolysis.

At the end of the experiments, surviving animals were killed by an overdose of ether and standardized autopsies were performed. An average of 8-10 step sections were prepared from the gastric and splenic lobes of the pancreas and 2-3 from the duodenal lobe, which means that the distance between each section was at most 1.5 mm. The histological preparations were stained with haematoxylin-erythrosin. The diagnostic criteria used are in accordance with those described by Pour and Wilson (6). Ductal proliferation was classified as slight (1-2 proliferations per low power field, 25 x magnification), moderate (2-4 proliferations per magnification field) or marked (>4 proliferations per magnification field) (fig. 2) and the degree of differentiation of cancers as high, moderate or poor (fig. 3). The pathologist made all histological examinations without knowledge of group association of the specimens.

RESULTS

In figure 4 the number of spontaneous deaths of animals in group C is demonstrated. At least 90% of animals treated with BHP for 18, 24 or 30 weeks died before the 52nd week of the study. All those which died after 24 and 30 weekly BHP-injections had pancreatic cancer, whereas in about half of the animals dying after 18 weeks of carcinogen treatment no cancers were detectable. It is notable that all animals with pancreatic cancer but three died without widespread metastases of the disease. The mortality at 52 weeks was 10 and 40% for the C subgroups treated with BHP for 6 and 12 weeks, respectively; all of the histologically evaluable animals dying a spontaneous death suffered from cancer.

The development of ductal hyperplasia was as demonstrated in figure 5 dose-dependent both in groups A, B and C. It was further obvious - especially at the accumulated lower carcinogen doses - that the ductal hyperplasia progressed with time.

A dose-dependency of the frequency of tumor-bearing animals was demonstrated in groups A and B but was not apparent in group C where all the animals but one given the lowest carcinogen dose were tumor-bearing after 52 weeks (fig. 6). Within the dose-range used the development of cancer was dose-dependent in all three groups (A, B and C). However, it seemed as if time was a less important factor than the accumulated dose for the development of malignant than for benign tumors (fig. 6). The continuous development of tumor contradicts any reversibility of regression of induced tumors. It should also be mentioned that all tumors were of ductal appearance.

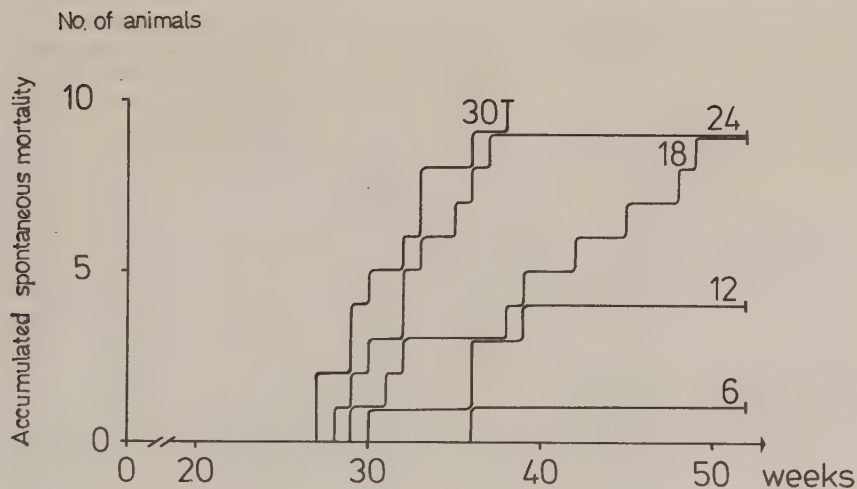


Fig. 4. Spontaneous death rate during the first 52 weeks after the start of the experiment in hamsters given weekly injections of BOP. For details see group C in Material and Methods and Fig. 1.

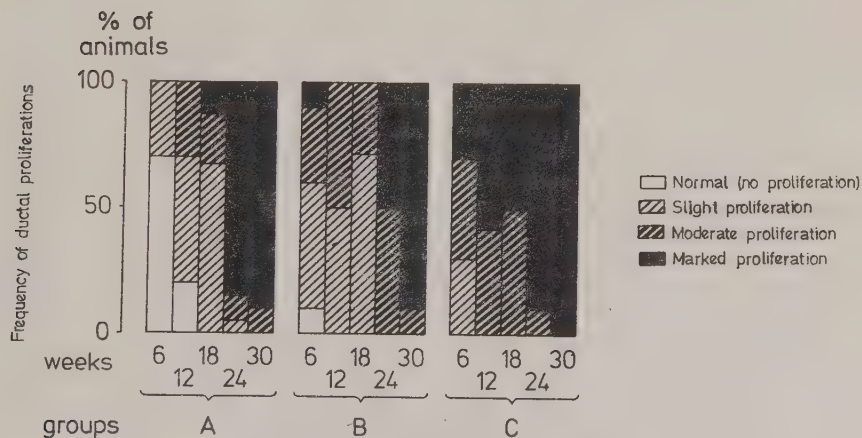


Fig. 5. Influence of accumulated carcinogen dose and of time on the development of ductal hyperplasia.

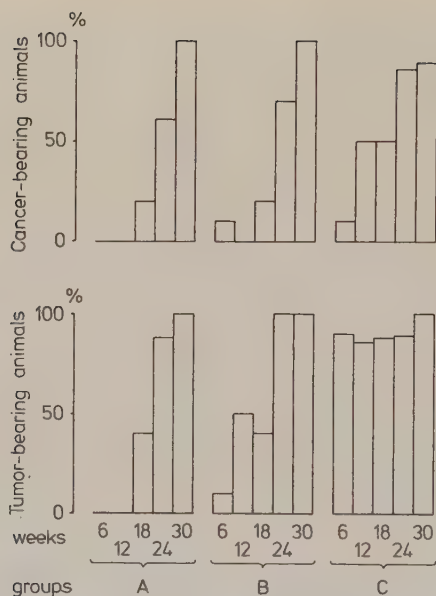


Fig. 6. Influence of accumulated dose of the carcinogen and of time on the frequency of cancer-bearing and tumor-bearing animals.

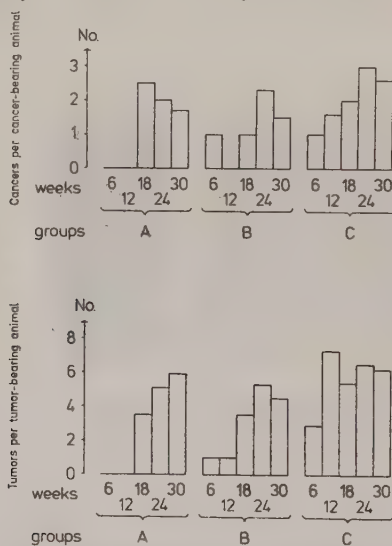


Fig. 7. Influence of accumulated dose of the carcinogen and of time on the frequency of cancers per cancer-bearing animal and tumors per tumor-bearing animal.

The number of tumors per tumor-bearing animal and number of cancers per cancer-bearing animal were related to the dose of carcinogen given as well as to time although these relationships seem to be weaker than was found for the severity of ductal hyperplasia and for tumor frequency (fig. 7). A confluence of several tumors may theoretically be a source of errors when using the number of tumors as a parameter of tumor progress. However, in this study the diameter of all benign tumors was 2 mm or less and so was the diameter in 82% of the cancers. Rarely the tumors were in close contact with each other and at no occasion there were difficulties to decide if a tumor was to be regarded as a single one, or as a conglomerate of more tumors. Although the size of the cancers tended to increase time, only 9 of the 135 cancers - all in hamsters given BHP for 30 weeks - were 5 mm or larger. Therefore, the number of benign tumors and cancers was regarded as a reliable reflexion of tumor development.

The histological degree of differentiation of the cancers showed a tendency to become lower with increased carcinogen dose whereas time in this respect was without importance (Table I). However, it was further found that tumors from different parts of the gland (gastric, splenic and duodenal lobe) displayed varying degree of differentiation in about 4% of the animals.

Table I. Degree of differentiation of pancreatic cancers as related to accumulated dose of the carcinogen and to time. For details see Material and Methods.

Group	No. of animals evaluable	Degree of differentiation of cancers		
		High	Moderate	Low
A 6	10			
B 6	9	1		
C 6	10	1		
A12	10			
B 12	10			
C 12	8	5	3	
A 18	15	3	2	
B 18	10	2		
C 18	8	7	1	
A 24	18	20	2	
B 24	10	11	5	
C 24	9	17		1
A 30	9	7	9	1
B 30	10	10	5	2
C 30	9	8	12	3

DISCUSSION

It has been shown by sequential histological studies that the nitroso compound BHP causes initial proliferation and distension of ductules due to primary hyperplasia of the ductular cells followed by metaplasia, atypia and malignant transformation (3). In the present study the influence of accumulated dose of the carcinogen and of time was systematically investigated. The development of ductal (ductular) hyperplasia, number of tumor-bearing animals, number of cancer-bearing animals, number of tumors per tumor-bearing animal and the degree of differentiation of cancers were found to be related to the accumulated dose of the carcinogen. The duration of the period between the carcinogen administration and examination (spontaneous death or sacrifice) was another factor of importance for the development of the same histological changes except for the degree of differentiation of malignant tumors. This relationship was, however, clearly evident only at the lowest accumulated carcinogen doses (6, 12 and 18 weekly carcinogen injections).

No attempts were made with single exposures of BHP as this has been shown not to induce pancreatic tumors (6). This is contrary to what has been reported after a single injection of the newer carcinogen N-nitrosobis(2-oxypropyl)amine (BOP) which led to pancreatic adenocarcinomas in 11/29 of the animals after a latency of 38 ± 10 weeks and a dose of 10 mg/kg body weight (5).

Most studies using the hamster model for experimental pancreatic carcinoma have hitherto dealt with the search for newer and more refined nitroso carcinogens, with carcinogen metabolism and with the histogenesis of the tumors. There is, however, also a need to use the model for in vivo studies of different possible risk factors for human pancreatic cancer as well as therapeutic modalities. One pre-requisite for such studies is good accuracy of the model to predict tumor-frequency when using a certain dose of carcinogen given for a certain time in experiments of a certain duration. The results of the present study may help to design such studies as it showed tumor-frequency to be determined by the dose of carcinogen as well as by the duration of the experimental period. Further the degree of differentiation of malignant tumors was dose-dependent as was mortality rate. It is clear from the present results and from those of others (4,9) that once carcinogenesis is initiated, it continues without signs of regression. In order to allow any conclusions from this kind of experiments it seems important that each laboratory elaborates its own dose- and time-response experiments, and continues to include adequate control groups whenever possible. Today the results of similar studies hitherto have been surprisingly different, e.g. with regard to tumor frequency (3,7,8). This is especially important as new nitroso compounds recurrently are being presented (4). Other reasons for the divergent results may be different sensibility of different hamster strains to the carcinogen and different principles for the selection of and number of sections prepared for histological examination. Therefore it is of utmost importance that the experiments are done in a highly standardized way, otherwise evaluation of the results and comparison of results from different laboratories are rendered difficult or impossible.

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CHANGES IN PANCREATIC DIGESTIVE AND LYSOSOMAL ENZYMES
DURING INDUCED PANCREATIC CARCINOGENESIS

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Running title: Lysosomal enzymes in experimental
pancreatic carcinoma

Summary

The activities of amylase and four acid hydrolases thought to be of lysosomal origin were measured in pancreatic tissue in the Syrian golden hamster during nitrosamine-induced (N-nitrosobis(2-oxypropyl)amine, BOP) pancreatic carcinogenesis. Amylase activity decreased after 18 weeks of treatment. β -hexosaminidase and β -glucuronidase increased markedly after 6, 12 and 18 weeks, whereas α -mannosidase and acid phosphatase were unaffected throughout the experiment. The changes in lysosomal enzyme activities were found prior to the development of pancreatic cancer. When ratios between β -hexosaminidase, β -glucuronidase and α -mannosidase on one hand and amylase on the other hand were calculated the divergent changes in digestive and lysosomal enzymes were even more pronounced. Our findings suggest that the increased secretion in pancreatic juice of certain lysosomal enzymes reported in hamsters developing pancreatic cancer is caused by an increased production and release of such enzymes within pancreatic tissue.

Key words: Induced pancreatic cancer, Syrian golden hamster, lysosomal enzymes, amylase, pancreatic tissue.

It has been shown by Rinderknecht et al (1982) that the pancreatic carcinogen N-nitrosobis(2-oxypropyl)amine (BOP) causes pancreatic secretory abnormalities in the Syrian golden hamster which precede the appearance of pancreatic malignancies. A striking decrease in flow rate and trypsin and chymotrypsin output was noted whereas on the other hand the output of lysosomal enzymes increased markedly. Similar findings have recently been reported by the same group (Rinderknecht, Renner, Stace, 1983) in patients with pancreatic cancer. Studies by Grant et al (1978) showed that pancreatic adenocarcinoma tissue does not produce digestive enzymes. It is further known that experimental (Hultberg, Mitelman, 1977) and human (Poole, 1973) tumors contain higher amounts of lysosomal enzymes than their tissues of origin.

The purpose of this study was to further elucidate the cause of the divergent influence of pancreatic carcinogens on pancreatic digestive and lysosomal enzymes, respectively, in the Syrian golden hamster.

Material and method

Inbred eight weeks old Syrian golden hamsters weighing on an average 100 g at the start of the experiments were used. In all experiments equal representations of both sexes were included. During the experimental period the animals were housed in groups of two in plastic cages and kept on a standard pellet diet. The animals had free access to tap water. In groups of six the hamsters were injected subcutaneously (0.5 ml) once weekly for 6, 12 or 18 weeks with N-nitrosobis(2-oxypropyl)amine (BOP) purchased from Ash-Stevens Co., Detroit, Michigan. One week after the last injection the animals were sacrificed by an overdose of ether. The pancreas was rapidly removed, freed of fat and connective tissue and frozen. It was later homogenized and analysed for protein and enzyme content (vide infra). Control animals in groups of six were given saline injections (0.5 ml) subcutaneously once weekly for 0, 6, 12 and 18 weeks, respectively.

Enzyme assays were performed on crude tissue homogenates. The tissues were homogenized in ice-cold distilled and deionized water (1/100) by using an all glass Potter-Elvehjem homogenizer. Protein in homogenates was assayed by the method of Lowry et al (1951) with a suitable albumin solution (Kabi, Stockholm, Sweden) as protein standard. β -Hexosaminidase (E.C. 3.2.1.30) and α -mannosidase (E.C. 3.2.1.24) were analysed as described before. Acid phosphatase (E.C. 3.1.3.2); 50 μ l of 1 mmol/l solution of 4-methylumbelliferyl-phosphate were incubated for 30 minutes with 50 μ l 1 mmol/l citratbuffer pH 5.0 and 25 μ l homogenate. β -glucuronidase (E.C. 3.2.1.31); 100 μ l of 1 mmol/l solution of 4-methylumbelliferyl- β -D-glucuronide in 100 mmol/l citrat-phosphate buffer pH 4.5 were incubated for 30 minutes with 25 μ l homogenate. The reaction

both for assay of acid phosphatase and β -glucuronidase was stopped by the addition of 3 ml of 0.2 mol/l glycine buffer pH 10.7. The fluorescence of 4-methylumbelliferone was read as described before (Hultberg, Lindsten, Sjöblad, 1976).

Amylase (E. C. 3.2.1.1) was assayed with the Phadebas-amylase test (Pharmacia, Uppsala, Sweden).

Results

The frequency of tumor-bearing animals was 25%, 83% and 100% after 6, 12 and 18 weeks of carcinogen administration, respectively. At the same time intervals 0%, 25% and 83% of the animals had developed cancers.

The pancreatic activities of α -mannosidase and acid phosphatase were uninfluenced by administration of the carcinogen (table I). By contrast the activities of β -hexosaminidase and β -glucuronidase in pancreatic tissue exhibited remarkably high levels especially at 12 and 18 weeks (Fig. 1 and 2). It is further obvious that the levels of pancreatic lysosomal enzymes in the control group did not change with time (table I, Fig. 1 and 2).

Table I. Activities of α -mannosidase and acid phosphatase in homogenates of pancreatic tissue from animals with BOP or saline for 0, 6, 12 or 18 weeks, respectively. Values are given as $\mu\text{mol/minute/gram protein}$ (mean $\pm$ SD). In no respect were any statistical differences found.

Weeks	α -mannosidase		Acid phosphatase	
	Saline	BOP	Saline	BOP
0	0.15 $\pm$ 0.04	-	1.78 $\pm$ 0.51	-
6	0.22 $\pm$ 0.05	0.22 $\pm$ 0.08	2.09 $\pm$ 0.91	1.64 $\pm$ 0.35
12	0.14 $\pm$ 0.05	0.26 $\pm$ 0.04	1.46 $\pm$ 0.45	2.22 $\pm$ 0.31
18	0.16 $\pm$ 0.02	0.20 $\pm$ 0.05	1.56 $\pm$ 0.65	1.30 $\pm$ 0.20

A decrease of the specific pancreatic amylase activity was obvious after 18 weeks of carcinogen administration but not after 6 and 12 weeks (Fig. 3). Also, a statistically significant fall by 20% in pancreatic protein concentration (data not shown) was found after 18 weeks. When calculating ratios

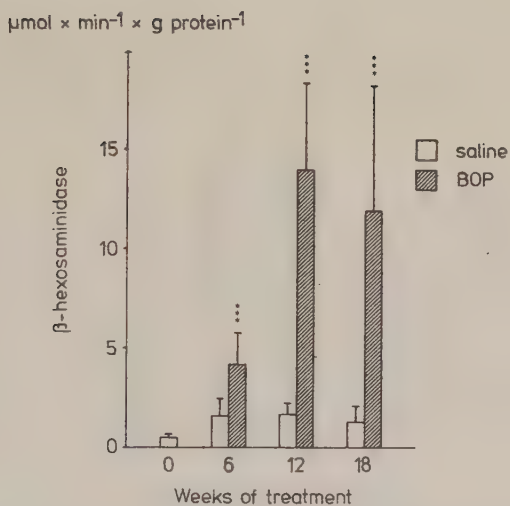


Fig. 1. Activity of β -hexosaminidase in homogenates of pancreatic tissue after 0, 6, 12 or 18 weeks treatment with BOP or saline.

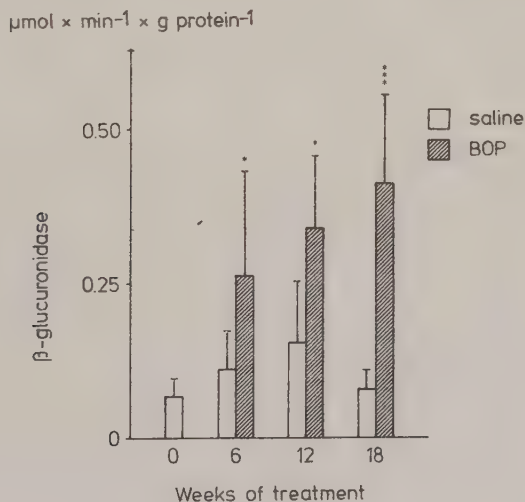


Fig 2. Activity of β -glucuronidase in homogenates of pancreatic tissue after 0, 6, 12 or 18 weeks treatment with BOP or saline.

$\mu\text{mol} \times \text{min}^{-1} \times \text{g protein}^{-1}$

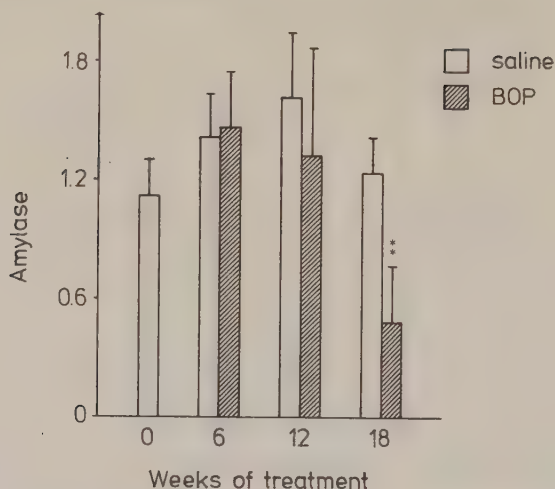


Fig. 3. Activity of amylase in homogenates of pancreatic tissue after 0, 6, 12 or 18 weeks treatment with BOP or saline.

Table II. Ratios of activities of lysosomal enzymes (α -mannosidase, β -hexosaminidase, β -glucuronidase and acid phosphatase) to amylase in pancreatic tissue of hamsters given BOP or saline for 0, 6, 12 or 18 weeks (mean $\pm$ SD).

Weeks	α -mannosidase/amylase		β -hexosaminidase/amylase		β -glucuronidase/amylase		Acid phosphatase/amylase	
	Saline	BOP	Saline	BOP	Saline	BOP	Saline	BOP
0	3.49 $\pm$ 1.25	-	1.15 $\pm$ 0.66	-	1.82 $\pm$ 0.82	-	3.56 $\pm$ 1.48	-
6	3.74 $\pm$ 0.99	3.93 $\pm$ 2.15	2.69 $\pm$ 1.54	3.60 $\pm$ 1.63	1.82 $\pm$ 0.86	*4.65 $\pm$ 2.75	3.42 $\pm$ 1.24	2.56 $\pm$ 1.06
12	2.82 $\pm$ 1.54	3.71 $\pm$ 1.05	2.63 $\pm$ 1.20	*7.53 $\pm$ 3.86	2.56 $\pm$ 1.78	*5.34 $\pm$ 3.03	2.86 $\pm$ 1.38	3.14 $\pm$ 0.85
18	3.05 $\pm$ 0.43	*8.20 $\pm$ 3.30	2.75 $\pm$ 1.19	*10.18 $\pm$ 5.53	1.54 $\pm$ 0.34	*18.11 $\pm$ 9.32	3.40 $\pm$ 2.28	5.45 $\pm$ 2.10

of lysosomal enzymes to amylase the diametrically opposite effect of the carcinogen on β -hexosaminidase and β -glucuronidase on one hand and amylase on the other hand was even more obvious. Also, the ratio of α -mannosidase to amylase was statistically increased in the carcinogen group as compared to the controls after 18 weeks (table II).

Discussion

The activities of four acid hydrolases thought to be of lysosomal origin were measured in pancreatic tissue during induction of ductal pancreatic cancer using the nitrosamine compound BOP. A dramatic increase in β -hexosaminidase and β -glucuronidase levels were found; this increase was noted before the appearance of malignant pancreatic tumors. On the other hand α -mannosidase and acid phosphatase activities were unaffected by the carcinogen. The activity of amylase and the protein concentration in pancreatic tissue was found to decrease after 18 weeks of carcinogen administration by comparison with the controls.

Rinderknecht et al (1982) have recently reported pancreatic secretory abnormalities in hamsters given the same pancreatic carcinogen as used in the present study. They found a striking decrease in flow rate and decreased output of trypsin and trypsinogen whereas the output of β -glucuronidase and α -glucosidase in pancreatic juice increased markedly. These changes were observed before the appearance of histologically recognizable pancreatic tumors. Reber and co-workers (1977) previously reported similar pre-malignant changes - decrease in flow rate, digestive enzymes and bicarbonate secretion - after administration of another pancreatic carcinogen (N-nitrosobis(2-hydroxypropyl)amine, BHP, to hamsters. The findings of Rinderknecht et al (1982) and Reber et al (1977) are reported and extended by the results of the present study. Thus, it seems probable that the increase in pancreatic secretion of certain acid hydrolases during pancreatic carcinogenesis is caused by an increased production and release within pancreatic tissue. The origin of acid hydrolases in pancreatic juice and pancreatic tissue is not known at present. Indirect evidence suggests that at least some of them may originate from ductular as well as acinar cells (Rinderknecht, Renner, Koyama, 1979). It is also possible that the tumor tissue itself is capable to produce lysosomal enzymes (Poole, 1973). The most probable explanation is, however, that inflammatory processes associated with the carcinogen administration are responsible for the increase in pancreatic lysosomal enzymes. It is known that activated macrophages in inflammatory lesions secrete excessive amounts of lysosomal enzymes (Davies, Bonney, 1979). It is further known that the amount of various lysosomal enzymes is increased to a different degree and that some of the enzymes (e.g. α -mannosidase) are mainly unaffected when macrophages are activated (Goodrum,

Spitznagel, 1982). This is compatible with the finding of the present study where two of the lysosomal enzymes measured were markedly increased in pancreatic tissue, whereas the other two enzymes were not changed at all.

The finding of a decrease in pancreatic protein concentration and in pancreatic amylase activity in tumor(cancer)-bearing pancreatic glands is supported by the study of Grant et al (1978). He reported that pancreatic cancer tissue did not synthesize any digestive enzymes. Whether this is a consequence of the replacement of normal pancreatic tissue by tumor tissue or whether the tumor cell elaborates factors which depress zymogen production is not known at present.

In healthy volunteers Rinderknecht et al (1979) have found that numerous lysosomal enzymes are secreted in pure pancreatic juice in a similar manner as digestive enzymes after stimulation with cholecystokinin. This is contradictory to their findings in hamsters (Rinderknecht, Habermel, Maset et al, 1982) and patients with pancreatic cancer (Rinderknecht, Renner, Stace, 1983) that the secretion of certain lysosomal enzymes does not parallel that of digestive enzymes after hormonal stimulation. Their findings in pancreatic juice of a depression of digestive enzymes whereas lysosomal enzymes are markedly elevated or remain normal is, however, in good accordance with our findings in pancreatic tissue from hamsters developing pancreatic cancer. In this context it is of interest to mention that although stimulation with cholecystokinin causes a reduced secretory response of protein and digestive enzymes in hamsters with induced pancreatic cancer as compared to normal hamsters (Reber, Johnsson, Montgomery, Carl, 1977; Doria, Mosley, Reber, 1981) long-term administration of the cholecystokinin-analogue caerulein had as strong trophic effect on the pancreas in hamsters developing tumors induced by BOP as in normal hamsters (Andrén-Sandberg, Dawiskiba, Ihse, 1983).

In summary, the results of the present study show that induction of pancreatic cancer in the hamster leads to diametrically opposite changes in certain lysosomal enzymes and digestive enzymes (amylase) within pancreatic tissue. These changes parallel those described by Rinderknecht et al (1982) in the pancreatic secretion using the same animal model. Our findings suggest that the increased secretion in pancreatic juice of certain lysosomal enzymes in animals developing pancreatic cancer is caused by an increased production and release of such enzymes within pancreatic tissue.

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INFLUENCE OF N-NITROSOBIS(2-OXOPROPYL)AMINE (BOP)
ON PANCREATIC SECRETION IN THE SYRIAN GOLDEN HAMSTER

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Running title: Effects of carcinogens on pancreatic secretion

ABSTRACT

In the conscious Syrian golden hamster the acute effects of the pancreatic carcinogen N-nitrosobis(2-oxopropyl)amine (BOP) on unstimulated exocrine pancreatic secretion were studied. Due to the minute amounts of juice obtained when pure pancreatic juice was collected combined bile-pancreatic juice was sampled. In separate experiments it was found that the administration of BOP did not influence the secretion of pure bile (volume or protein output).

Both a single subcutaneous (10 mg/kg bw of 100 mg/kg bw) and a single intravenous (10 mg/kg bw) injection of BOP caused a reduction in secretory volume, and protein and amylase output from the bile-pancreatic fistulas. Also, in animals pre-treated with weekly BOP injections for 6 or 12 weeks, a similar decrease in secretory volume and protein output following BOP administration was found. Bicarbonate output was assayed, after a single subcutaneous BOP injection, and in animals pre-treated with BOP for 12 weeks, Contrary to the BOP effects on secretory volume and protein output, a clear-cut gradual increase in bicarbonate concentration was found after the BOP injection, therefore bicarbonate output was practically unaffected.

It is concluded that the administration of BOP causes early - disparate - functional changes in acinar and ductal pancreatic cells, respectively. The relationship between these functional changes and pancreatic carcinogenesis remains to be elucidated.

Key words: BOP; pancreatic secretion; Syrian golden hamster.

In the Syrian golden hamster subcutaneous injections of N-nitrosobis(2-oxopropyl)amine (BOP) induces a pancreatic cancer of ductal appearance (1). It has, however, recently been questioned whether the cancer is of ductal origin (2). Instead it has been suggested that the acinar cell is the cell of origin which under the influence of the carcinogen undergoes dedifferentiation to cells with ductal characteristics (2). Electron microscopically detectable morphological changes have been reported to appear within acinar as well as ductal cells as soon as 45 minutes after the administration of BOP (3). In the present study the effects of BOP administration on unstimulated exocrine pancreatic secretion was studied in the conscious hamster.

Material and Methods

Animals. Eight weeks old Syrian golden hamsters from own breeding, equal numbers of both sexes, and weighing about 100 g at the start of the experiments, were used. The animals were kept on a standard pellet diet since birth and till the evening before the secretory studies.

Surgical method. The animals were anaesthetised with intraperitoneal mebumalum (ACO, Stockholm, Sweden) and an abdominal incision was made. The bile-pancreatic duct was cannulated close to the duodenal wall with a silastic catheter with an inner diameter of 0.58 mm and a length of 15 cm (Dow Corning 602-105) and the catheter was fixed with its tip at most 2 mm from the duodenal wall with a silk ligature. When only bile was collected a catheter of similar type was introduced into the bile duct high up in the hilum of the liver.

The animals were kept in restraining cages and were allowed about 3 hours of surgical recovery before the start of the experiments. Food and water was withdrawn but 10 ml of a solution containing 1 part glucose 5.5%, and 1 part Ringer solution was injected subcutaneously in each animal.

Laboratory techniques. The bile-pancreatic juice and bile, respectively, was weighed and the volume calculated assuming an sp. grav. of 1.00 and its protein content determined as described by Lowry et al (4). Amylase activity was assayed using the Phadebas test (Pharmacia, Uppsala, Sweden) and bicarbonate indirectly from pH and $p\text{CO}_2$ (5). The secretion was collected on ice and in a closed sampling system.

Design of the study. After three one-hour collections of spontaneous bile-pancreatic secretion another seven one-hour samples were obtained after the BOP administration. In two groups 10 mg/kg bw BOP were given subcutaneously (group II) and intravenously (group III), respectively. In one group of hamsters 100 mg/kg bw BOP was given subcutaneously

(group IV). Control hamsters were given subcutaneous saline injections (group I). Also, two groups of hamsters were investigated after subcutaneous injections of 10 mg/kg bw of BOP following pre-treatment for 6 (group V) and 12 (group VI) weeks, respectively, by weekly subcutaneous injections of the same dose of BOP.

In another group of animals the effects on bile secretion of 10 mg/kg bw of BOP administered subcutaneously was studied. Bile was collected in the same way as described above for bile-pancreatic juice. Due to the small amounts of pancreatic juice obtained during the experiments either amylase or bicarbonate in general could be assayed in each sample. Therefore, the number of observations vary in figure 1.

Calculations. Statistical evaluations were done using the Student's t-test for paired observations or the Chi-square test.

Results

As the data of figs. 1-5 are expressed as the mean of the percentage changes of secretory volume, protein and bicarbonate output the numerical values are shown in table I. After 12 weekly BOP injections (group VI) the protein output was significantly decreased as compared to the basal output of the comparable groups I - IV put together (Table I).

Subcutaneous administration of saline did not influence secretory volume, protein- and bicarbonate output (data not shown). A single subcutaneous exposure to 10 mg/kg bw of BOP caused a significant decrease of protein and amylase output whereas bicarbonate output was apparently uninfluenced (figure 1). The same dose of BOP given intravenously caused, as shown in figure 2, a marked depression of secretory volume and a slight reduction of protein output. When BOP was given subcutaneously in a dose which was increased tenfold (100 mg/kg bw) both secretory volume and protein output was significantly depressed (figure 3).

As can be seen in figs. 4 and 5 secretory volume and protein output were significantly reduced after BOP administration also in animals pre-treated with the carcinogen for 6 or 12 weeks. In the group of animals pre-treated for 12 weeks bicarbonate output was assayed - as it was in group II - and found to be unaffected by the carcinogen administration (figure 5) whereas a significant increase in bicarbonate concentration was obvious.

It was also found that BOP did not cause any significant changes in secretory volume and protein output of pure bile (data not shown).

Table I. Basal levels of volume flow and protein output in hamsters used in the different experiments. Mean $\pm$ SD. Figures within parentheses denote number of hamsters.

Group	Acute treatment	Longterm treatment	Volume ml/h	Protein output mg/h	Bicarbonate output μ mol/h
I (3)	saline	-	0.44 $\pm$ 0.07	4.18 $\pm$ 1.74	30 $\pm$ 6
II (17)	10 mg/kg bw BOP s.c.	-	0.50 $\pm$ 0.09	3.92 $\pm$ 2.10	36 $\pm$ 6
III (5)	10 mg/kg bw BOP i.v.	-	0.48 $\pm$ 0.12	4.26 $\pm$ 1.71	-
IV (6)	100 mg/kg bw BOP s.c.	-	0.46 $\pm$ 0.08	4.30 $\pm$ 0.88	-
V (6)	10 mg/kg bw BOP s.c.	BOP 6 weeks	0.38 $\pm$ 0.16	3.62 $\pm$ 1.52	-
VI (5)	10 mg/kg bw BOP s.c.	BOP 12 weeks	0.33 $\pm$ 0.18	2.98 $\pm$ 2.33	22 $\pm$ 5

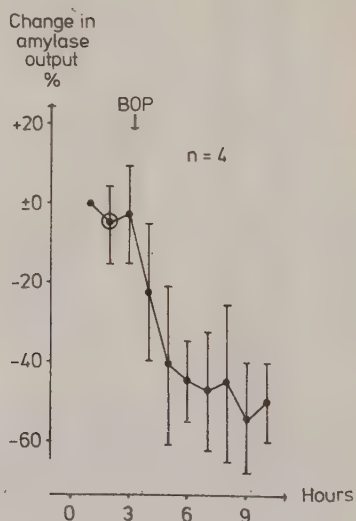
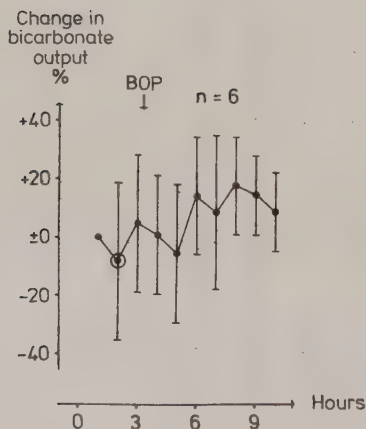
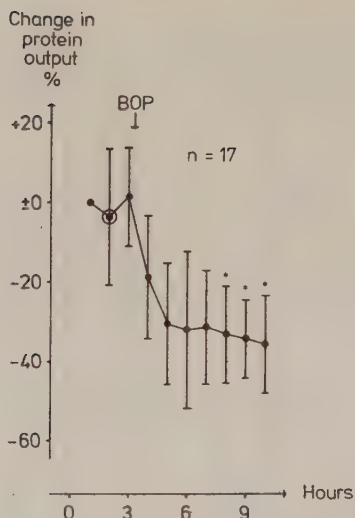
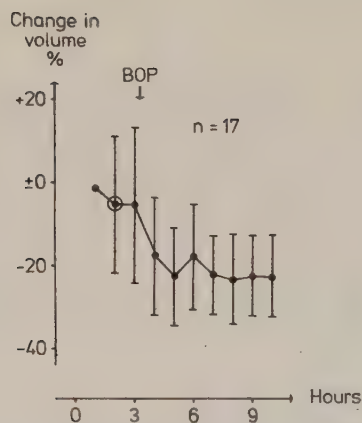


Fig. 1. Influence of a single subcutaneous injection of BOP (10 mg/kg bw) on secretory volume, protein output, bicarbonate output and amylase output in hamsters with bile-pancreatic fistulas. Due to the small number of animals ($n = 4$) the changes of amylase output was not evaluated statistically.

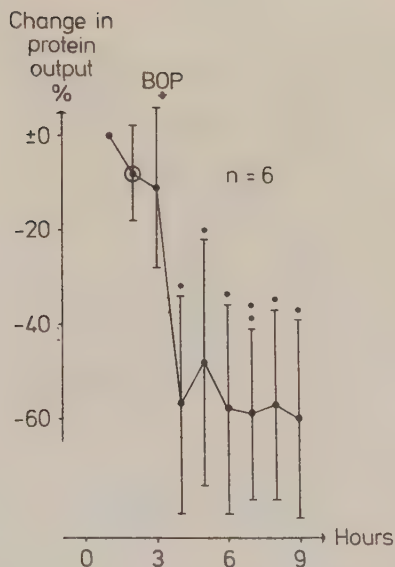
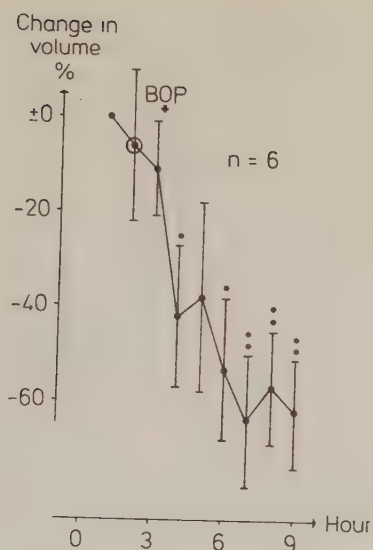


Fig. 2. Influence of a single intravenous injection of BOP (10 mg/kg bw) on secretory volume and protein output in hamsters with bile-pancreatic fistulas.

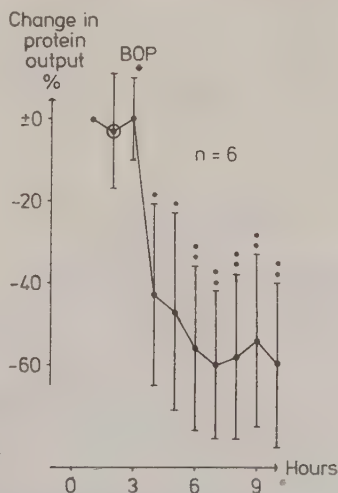
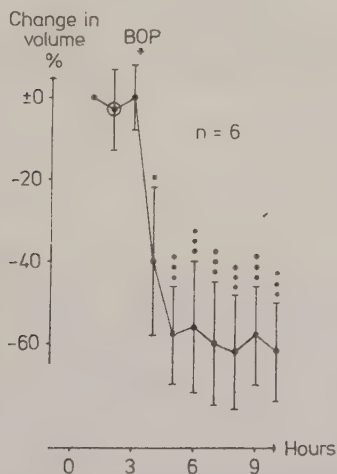


Fig. 3. Influence of a single subcutaneous injection of BOP (100 mg/kg bw) on secretory volume and protein output in hamsters with bile-pancreatic fistulas.

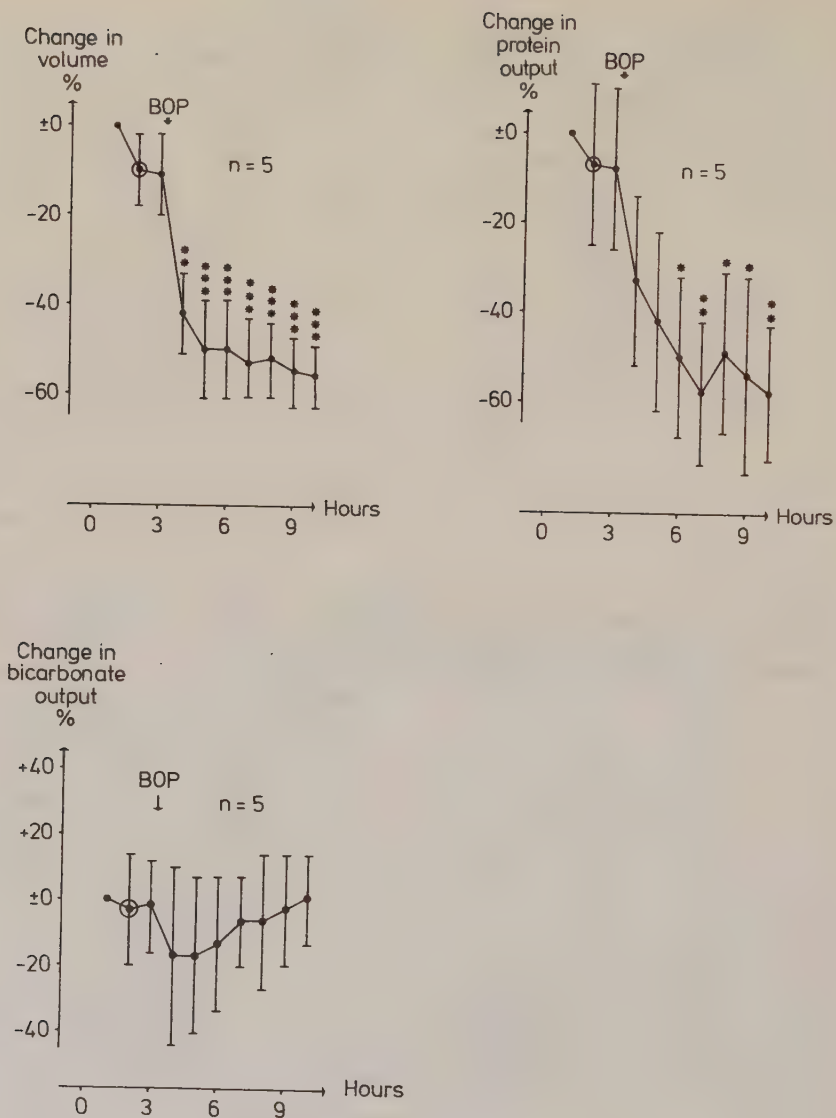


Fig. 4. Influence of subcutaneous BOP administration (10 mg/kg bw) on secretory volume and protein output in bile-pancreatic fistula hamsters pre-treated for 6 weeks with weekly BOP injections.

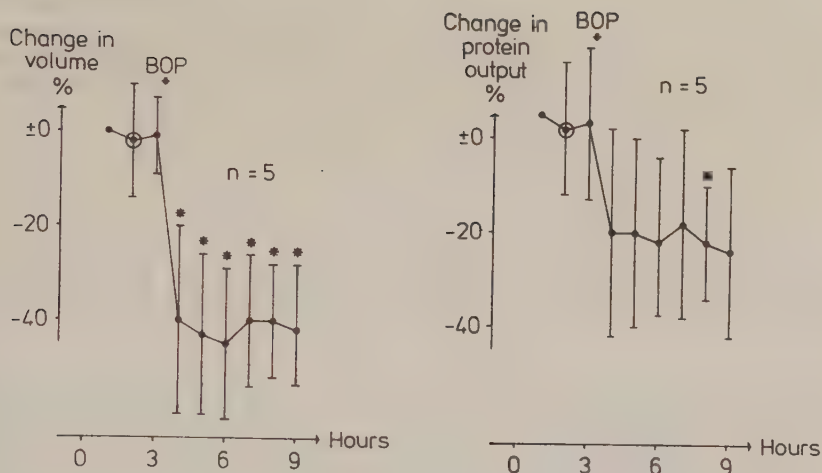


Fig. 5. Influence of subcutaneous BOP administration (10 mg/kg bw) on secretory volume, protein output and bicarbonate output in bile-pancreatic fistula hamsters pre-treated for 12 weeks with weekly BOP injections.

Discussion

The acute effects on pancreatic secretion of administration of the carcinogen BOP were studied in the Syrian golden hamster. After subcutaneous as well as intravenous BOP injections a marked depression of the secretory volume and protein output was found. Similar findings were made in identical experiments on hamsters pre-treated with six or twelve weekly injections of the carcinogen. On the other hand an increased bicarbonate concentration was found whereas bicarbonate output was unaffected by BOP administration in the two groups studied. In the control animals no effects were observed after subcutaneous saline injections. Similarly, the spontaneous secretory volume and protein output in a previous study using the same experimental design was found to be stable through 14 hours (6).

In the present study combined bile-pancreatic secretion was used in order to avoid problems related to the very small flow rate when pure pancreatic juice is collected. Pancreatic juice is a viscous substance - especially if the secretion is unstimulated as in this study - and bile may normally assist its passage down the common bile duct. Any errors in estimating pancreatic secretory rate brought about by diverting bile from the common bile duct were therefore avoided by collection of the combined bile-pancreatic juice. The small amounts of bicarbonate secreted by bile ducts or ductuli are quite negligible (7). Further, as protein output of pure bile was unaffected by BOP, and as amylase secretion in one of the experiments was found to closely parallel that of protein, we find it legitimate to regard the changes in protein output as a reflexion of pancreatic enzyme secretion.

A single dose of 10 mg/kg bw of BOP - the lowest carcinogen dose used in this study - has been shown to cause pancreatic tumors in more than half of the tested hamsters and after 6 weekly subcutaneous injections tumors were found in all of the animals (8). Although the BOP-induced pancreatic cancers, like the majority of human pancreatic cancers, are of ductal appearance, it has recently been questioned that they are of ductal origin (2). The results of the present study point to early changes of the acinar and ductal cell function after administration of BOP. Doria et al studied the effects of a single exposure to the carcinogen N-nitrosobis(2-hydroxypropyl)amine (BHP) and found depressed protein output immediately after carcinogen administration (9). As, further, the changes in protein (enzyme) as well as bicarbonate secretion in our study occurred as early as after 1-2 hours a direct effect of the carcinogen on the acinar and ductal cells, respectively, is most probable. Recently Levitt et al (10) reported unspecific ultrastructural changes of both acinar and ductal cells 4 hours after the administration of BHP whereas Ogrowsky and Wilson (3) found similar changes as early as 45 minutes after a BOP injection.

The relationships of early functional and structural changes in the acinar and ductal pancreatic cells to pancreatic carcinogenesis by BOP is not clear at the moment. The changes may reflect carcinogen metabolism. Recently, Reznik-Schuller et al (11) showed that eight hours after a single dose of ³H-N-Nitroso-2,6-di-methyl-morpholine (DMNM) the distribution of labelled carcinogen in the pancreas was confined exclusively to the acinar and ductal cells as found at electron microscope autoradiography. This is consistent with the idea that the activation of carcinogen can occur in the acinar and/or ductal cells. Further studies on early cellular events, preferably using acinar or ductal cell specific markers, are, however, of importance to elucidate if ductal pancreatic cancer is originating from malignant transformation of either acinar or ductal cells or both.

Acknowledgements

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EFFECT OF BILI-DIGESTIVE ANASTOMOSIS ON EXPERIMENTAL PANCREATIC CARCINOGENESIS

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Abstract. Hamsters were subjected to cholecysto-duodenostomy and a small suprapancreatic bile-duct resection in order to preclude bile reflux into the pancreatic ducts. The model was used to investigate the role of bile reflux in the pancreatic carcinogenic action of BHP (N-nitrosobis(2-hydroxypropyl)amine). The carcinogen was given by weekly injection for 20 or 24 weeks. No statistically significant effects of the bile deviation on tumour frequency were found at the end of either time. BHP per se did not induce tumours in any preferential area of the gland. The tumours thus were uniformly distributed. Corresponding distribution was found in the hamsters with choledochoduodenostomy. The histologic appearance of the pancreatic lesions was the same in hamsters with and without choledochoduodenostomy. The findings do not support the concept of bile reflux as an aetiological factor in experimental pancreatic carcinogenesis.

The incidence of pancreatic cancer is increasing in most countries. Studies on factors influencing pancreatic carcinogenesis have hitherto been impeded by lack of useful experimental models. Recently, however, a suitable method has been developed for induction of ductal pancreatic cancer in the Syrian golden hamster (Pour, Mohr, Cordesa, Althoff & Krüger, 1975; Pour, 1979).

In man most pancreatic cancers are located in the head of the gland (Cubilla & Fitzgerald, 1978). Reflux of bile into the pancreatic ducts has therefore been suggested as an aetiological factor in this disease (Wynder, Mabuchi, Maruchi & Fortner, 1973). Accumulation of induced pancreatic cancer to the head of the gland, around the collecting main ducts, has been claimed to occur in hamsters (Pour, Althoff & Takahasi, 1977; Takahasi, Pour & Althoff, 1977). Available results on the role of bile reflux in experimental pancreatic cancer are few and conflicting (Pour & Donnelly, 1978; Rückert, Klink & Klöppel, 1979). Thus, in contrast to Rückert et al., Pour & Donnelly found no evidence for influence of bile deviation on experimental pancreatic carcinogenesis.

The present study concerned the importance of bile reflux on the multiplicity, distribution and morphology of induced pancreatic tumours in hamsters.

MATERIALS AND METHODS

Inbred 8-weeks-old Syrian golden hamsters were used. Their average weight at the start of the experiments was 100 g. The sexes were equally represented in all experiments. During the experimental period the hamsters were housed in groups of 2 in plastic cages and were kept on a standard pellet diet with free access to tap water.

In 143 hamsters, BHP, N-nitrosobis(2-hydroxypropyl)amine, was given by subcutaneous injection weekly for 20 or 24 weeks. The dose (1 ml) was 125 mg/kg body weight in 0.9% saline solution. BHP was purchased from Ash Stevens Co., Detroit, Michigan, USA. In 48 of these 143 hamsters a bili-digestive shunt was created. Under ether anaesthesia the abdominal cavity was entered via an upper midline incision. The bile duct was identified and ligated and a short segment of it was resected just below the entrance of the cystic duct. A cholecystoduodenostomy was performed, using one layer of uninterrupted sutures. The anastomosis was made at least 15 mm below the entrance of the bile duct into the duodenum, in order to preclude bile reflux from the intestine. At the end of the operation 5 ml of a solution containing 1 part Ringer's solution and 1 part 5.5% glucose solution was deposited subcutaneously in each animal. As controls, 90 additional hamsters were given weekly subcutaneous injections of 1 ml saline solution.

At the end of the experiments, i.e. after 20 or 24 weeks, the hamsters were weighed and killed by ether overdose. Necropsy was performed according to a standardized schedule. The pancreas was removed and weighed. On average eight to ten step sections were prepared from the gastric and splenic lobes of the pancreas and two or three from the duodenal lobe, which means that the distance between each section was at most 1.5 mm. The histologic preparations were stained with haematoxylin-eosin. The diagnostic criteria were in accordance with those described by Pour & Wilson (1980).

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Table 1. Results of cholecystoduodenostomy (CD) in regard to body weight, pancreatic weight and tumour frequency in hamsters given weekly subcutaneous injections of BHP

Group	No. of hamsters	Body weight (g)	Pancreas (wet) weight (g)	Tumor-bearing hamsters (%)	No. of tumours per tumour-bearing hamster	No. of adenocarcinomas per tumour-bearing hamster
Untreated						
20 weeks	45	116±13	0.40±0.14	0	0	0
BHP						
20 weeks	45	119±17	0.39±0.09	56 (25/45)	2.24	0.72
BHP+CD						
20 weeks	26	107±17	0.37±0.11	38 (10/26)	2.20	0.86
Untreated						
24 weeks	45	131±17	0.48±0.14	0	0	0
BHP						
24 weeks	50	121±17	0.41±0.12	78* (39/50)	4.12*	1.28*
BHP+CD						
24 weeks	22	111±11	0.32±0.08	77** (17/22)	3.29**	1.53**

* Significantly different from BHP 20 weeks.

** Significantly different from BHP+CD 20 weeks.

The exact anatomic location of all the histologically demonstrated lesions was recorded on a diagram of the pancreas.

Statistical analysis of the recorded data was made with the Log-Odd ratio test.

RESULTS

The body weight of the hamsters and the pancreatic weight were uninfluenced by the BHP treatment. Nor were they influenced by the creation of cholecystoduodenostomy (Table 1).

After 20 weeks of BHP treatment, 56 per cent of the hamsters without cholecystoduodenostomy were tumour-bearing and after 24 weeks the incidence had risen to 78% (Table 1). The rise was

statistically significant. The BHP-treated hamsters with cholecystoduodenostomy also showed increasing tumour incidence with time. Although the numerical value after 20 weeks was lower in this latter group, the bile deviation per se did not give statistically significant change in the number of tumour-bearing animals (Table 1). The number of tumours per tumour-bearing animal was likewise uninfluenced by the cholecystoduodenostomy. It increased with time to the same extent as in the BHP-treated hamsters without surgery (Table 1).

There was no accumulation of tumours to the part of the gland corresponding to the pancreatic head. Instead, the distribution of tumours was uniform throughout the gland after both 20 and

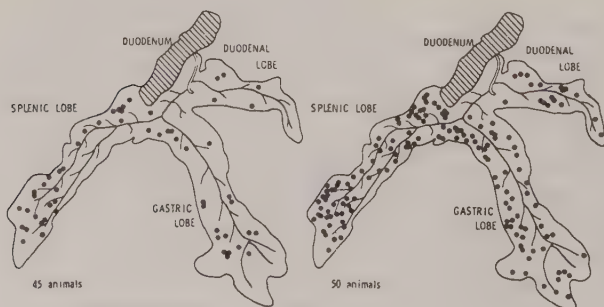


Fig. 1. Distribution of benign and malignant BHP-induced pancreatic tumours in hamsters without bile deviation. Left = 20 weeks of BHP treatment. Right = 24 weeks of BHP.

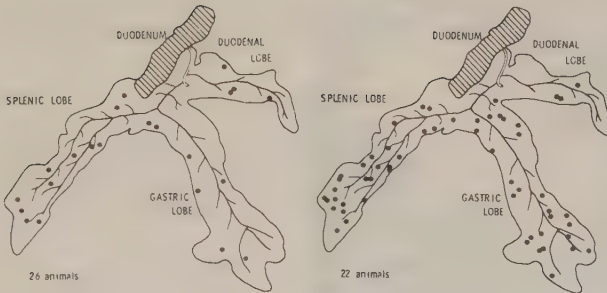


Fig. 2. Distribution of benign and malignant BHP-induced pancreatic tumours in hamsters with cholecystoduodenostomy. Left = 20 weeks of BHP treatment. Right = 24 weeks of BHP.

24 weeks of carcinogen administration (Fig. 1). Similar uniformity of distribution, with no preferential area, was found in the BHP-treated hamsters with cholecystoduodenostomy (Fig. 2). The patterns of distribution were similar for benign and for malignant lesions in all of the studied groups.

The proportion of adenocarcinomas was not affected by bile deviation at either time interval (Table I). The morphologic appearance of benign and of malignant tumours was not dependent of presence or absence of cholecystoduodenostomy. The degree of multicentricity thus was the same in experiments of corresponding duration. Ductal hyperplasia, cystadenoma (Fig. 3), papillary adenoma, solid adenoma (Fig. 4) and ductal adenocarcinoma (Fig. 5) showed similar occurrence in the hamsters with and in those without cholecystoduodenostomy.

DISCUSSION

Whether carcinogens reach the pancreas via reflux of bile or duodenal contents into the pancreatic ducts, or directly from the circulation, is not known. As most human pancreatic cancers originate in the head of the gland (Cubilla & Fitzgerald), bile reflux is often presented as a probable aetiological factor.

In the present study, deviation of the bile by means of a cholecystoduodenostomy did not significantly influence the frequency, distribution or morphology of BHP-induced pancreatic tumours in hamsters. The observations support and extend the report by Pour & Donnelly who, in a small number of hamsters, found no influence of bile

deviation on the pancreatic carcinogenic action of BOP (N-nitrosobis(2-oxypropyl)amine). Unfortunately, they did not use an adequate control group and, furthermore, the observation time differed significantly also within the groups. In contrast to the results in our study and to those described by Pour & Donnelly, Rückert et al. reported a markedly decreased tumour incidence after bili-digestive anastomosis in hamsters given BHP for 15 weeks. Like us, however, Rückert et al. found no accumulation of tumours to the 'head of the pancreas' after subcutaneous administration of carcinogen, but uniform distribution throughout the gland.

If bile reflux into the pancreatic ducts is important as a carcinogenic factor, the highest carcinogen concentration should occur along the intrapancreatic part of the bile duct, and therefore an accumulation of tumours would be expected in the pericholedochal area (Figs. 1 and 2). The decreased tumour frequency throughout the gland that Rückert et al. found after bile deviation is therefore difficult to explain.

In the present study a surgically created bypass of bile into the intestine prevented possible bile-borne carcinogens from reaching the pancreatic ducts. The frequency, distribution and morphology of the induced tumours, however, were uninfluenced by the bile deviation. The results therefore do not suggest that bile reflux into the pancreatic ducts is an aetiological factor in experimental pancreatic carcinogenesis. Further, it is logical to assume that reflux from the duodenum of, for instance, dietary carcinogens would result in a higher frequency of tumours around the distal common bile-pancreatic duct. This was not the

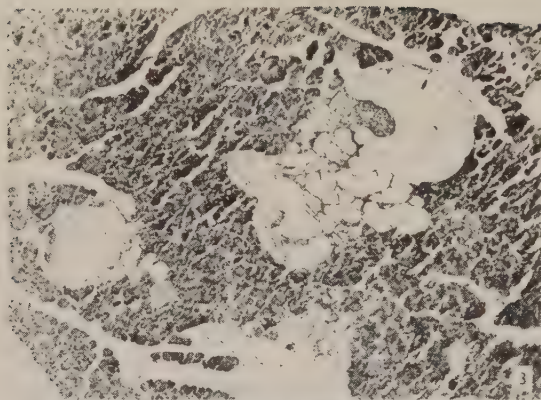


Fig. 3. Intrainsular cystadenoma.

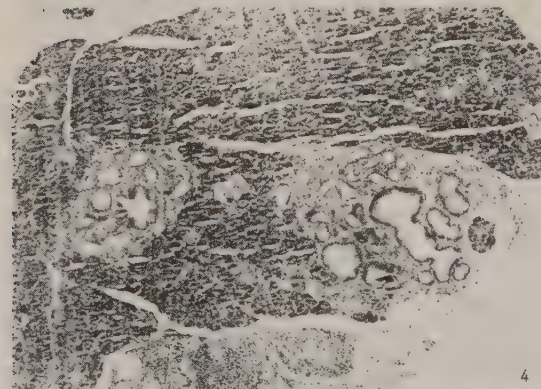


Fig. 4. Two solid adenomas with regular epithelium.

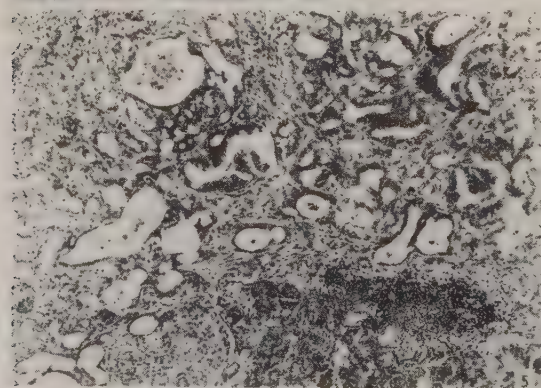


Fig. 5. Ductal adenocarcinoma growing beneath an insula. Upper left: normal exocrine pancreatic tissue.

case in the present study. Consequently, it seems probable that in hamster the carcinogen reaches the pancreas via the blood supply.

ACKNOWLEDGEMENTS

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Regulatory Effects on the Pancreas of Intraduodenal Pancreatic Juice and Trypsin in the Syrian Golden Hamster

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Andrén-Sandberg Å, Ihse I. Regulatory effects on the pancreas of intraduodenal pancreatic juice and trypsin in the Syrian golden hamster. *Scand J Gastroenterol* 1983; 18, 697–706.

The effect of intraduodenal trypsin activity on pancreatic exocrine secretion was studied in conscious Syrian golden hamsters provided with bile-pancreatic fistulae. The secretion (secretory volume, amylase and protein output) was stable during a collection period of 14 h without any duodenal infusions. Infusion into the duodenum of bicarbonate or bile did not affect the secretion. When, however, bile-pancreatic juice or trypsin was administered intraduodenally, a marked depression of amylase and protein output was found. After addition of trypsin inhibitor—in a dose sufficient to eliminate all trypsin activity—to either of the two infusates the secretion was restored to the initial values. In a long-term experiment (10 days) repeated subcutaneous injections of cholecystokinin caused a significant increase of pancreatic protein and amylase content in the hamster. Oral trypsin inhibitor administration for 10 days had similar, although not so pronounced effects. Subcutaneous secretin administration was without effect in this respect. The results show that pancreatic enzyme secretion in the Syrian golden hamster is controlled by a negative feedback regulation exerted by intraluminal trypsin. The findings also suggest that both cholecystokinin and orally administered trypsin inhibitor exert trophic effects on the pancreas.

Key words: Cholecystokinin; feed-back regulation; hamster; pancreatic enzyme secretion; secretin; trophic effects; trypsin; trypsin inhibitor

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In 1958 Grossman (1) observed hypersecretion in rats with the pancreatic juice deviated from the intestine via a chronic pancreatic fistula. Later it was shown in acute experiments that pancreatic secretion in the rat is controlled by a negative feedback regulation exerted by intraluminal trypsin (2, 3). A similar regulatory mechanism seems to exist in the pig (4), whereas its presence in man still is a matter of dispute (5–7). It is further well established that long-term oral administration of trypsin inhibitor causes a hypertrophy of the pancreas in the rat, probably as a result of excessive stimulation of the pancreas due to sustained release of trophic hormones or factors from the intestinal mucosa (8). In the rat exogenously administered cholecystokinin (CCK) (9) and gas-

trin (10) are known to have trophic effects on the pancreas.

Hitherto, studies on pancreatic carcinogenesis have been hampered by a lack of useful experimental methods. Recently, however, Pour et al. (11) have shown that certain nitrosamine derivatives cause a ductal pancreatic adenocarcinoma in the Syrian golden hamster which has several similarities with the human pancreatic cancer. Since it has been suggested that regenerating tissue may have an increased susceptibility to carcinogens (12), we found it important to investigate whether a feedback mechanism exerted by intraluminal trypsin also exists in the hamster. Further, studies on possible trophic effects of oral trypsin inhibitor administration and of CCK on the ham-

ster pancreas were regarded of interest. Thus, the present study is to be looked upon as part of a series of experiments dealing with the possible effects of trophic hormones or factors on pancreatic carcinogenesis in the Syrian golden hamster.

MATERIALS AND METHODS

Animals

Syrian golden hamsters, equal numbers of both sexes, weighing 110–130 g, were used. The animals were bred by us and were 8–10 weeks old. Only one experiment was performed on each animal.

Surgical methods

Immediately before surgery food and water were withdrawn. Intraperitoneal Mebumalum (ACO, Stockholm, Sweden) was used for anesthesia. An abdominal incision was made, exposing the duodenum and the bile-pancreatic duct. A silastic catheter with an inner diameter of 0.58 mm and a length of 15 cm (Dow Corning 602–105) was introduced directly into the bile-pancreatic duct close to the duodenum and secured in place with a silk ligature. The tip of the catheter was placed no more than 3 mm from the duodenal wall. Another catheter of the same type was inserted into the duodenum just where the bile-pancreatic duct enters the intestine. Both catheters were brought out through separate stab incisions in the loin. At the end of the operation each animal was given a subcutaneous injection of 10 ml of a solution containing one part Ringer solution and one part 5.5% glucose solution. The animals were kept in restraining cages and were allowed to recover for about 3 h after surgery before the experiments were started. All experiments were performed on conscious animals. During the experimental period food and water were withheld from the animals. Animals in which surgical complications occurred were excluded from the study.

Intestinal infusion substances

Porcine crystalline trypsin (A/S Novo, Copenhagen, Denmark) was dissolved in 0.05 M

NaHCO₃. Highly purified bovine lung trypsin inhibitor (Trasylol®) was obtained from Bayer AG, Leverkusen, FRG, and dissolved in bile-pancreatic juice or the trypsin-NaHCO₃ solution. In ancillary experiments 0.5 mg of the inhibitor completely abolished the tryptic activity of 1 mg of the trypsin used.

Bile and bile-pancreatic juice used for infusion were collected on ice from hamsters provided with bile duct or bile-pancreatic duct fistulae and immediately frozen at -20°C. The bile—used in the experiments described below—was found to be free from trypsin or trypsinogen.

Laboratory techniques

The volume of the bile-pancreatic juice was measured. Protein was assayed in accordance with the method described by Lowry et al. (13). Amylase was determined with the Phadebas test, a gift from Pharmacia, Uppsala, Sweden.

Design of the studies on pancreatic secretion

In *experiment I* the spontaneous bile-pancreatic secretion was studied in 1-h samples collected during 14 h in eight animals. No intestinal infusions were given.

In *experiment II* the influence of intestinal bicarbonate (0.05 M) infusion (0.5 ml/h) on pancreatic enzyme secretion was studied in three hamsters.

In *experiment III* the effect on pancreatic secretion of reintroduction of bile was investigated. Seven hamsters were used.

In *experiment IV* bile-pancreatic juice (0.5 ml/h) was reintroduced into the duodenum and the influence on pancreatic secretion studied in six hamsters.

In *experiment V* the design was identical to that of experiment IV except that 1.0 ml/h of bile-pancreatic juice was used in six hamsters.

In *experiment VI* trypsin inhibitor (Trasylol®; 50,000 kallikrein inhibitory units (KIU)/ml) was added to bile-pancreatic infusates (0.5 ml/h) 3 h after the start of the bile-pancreatic juice infusions. Five hamsters were studied.

In *experiment VII* trypsin (2.5 mg/ml) was infused (0.5 ml/h) into the duodenum and the

effects on pancreatic secretion studied in six hamsters.

In *experiment VIII* the design was similar to that of *experiment VII*, but after 3 h of trypsin infusion trypsin inhibitor (Trasylol®; 50,000 KIU/ml) was added to the infusate and the secretion collected for another 6 h in six hamsters.

The bile-pancreatic secretion was collected in small tubes on ice at 60-min intervals. The samples were immediately frozen at -20°C and analyzed in duplicate or triplicate within 4 weeks.

Design of the study on long-term effects

In the long-term experiment (10 days) 8-week-old Syrian golden hamsters were used. Twenty-five animals were given 0.9% saline (0.5 ml) by subcutaneous injection twice daily for 10 days. Fifteen animals were given 2 clinical units (CU) of secretin (Kabi-Vitrum, Stockholm, Sweden) in the same manner and same volume as saline twice daily for 10 days. Another 15 animals had subcutaneous injections of 2 Ivy dog units (IDU) of CCK (Kabi-Vitrum), using exactly the same schedule as for secretin. Finally, 10 hamsters were given 50,000 KIU of the bovine lung trypsin inhibitor (Trasylol®) dissolved in 0.5 ml of 0.9% saline by gastric intubation twice daily. After 10 days the animals were killed—20 h after the last injection or gastric intubation—and the pancreas rapidly removed and freed of connective tissue and lymph nodes and its wet weight measured. The pancreatic glands were homog-

enized in a Sorvall homogenizer, and their protein content and amylase activity were determined.

Calculations

Student's *t* test for paired observations was used in the infusion studies and Fisher's exact test when calculating the results of the long-term experiment.

RESULTS

Studies of pancreatic secretion

When bile-pancreatic juice was collected for 14 h (1-h samples) without any intestinal infusions, the secretion was stable throughout the experimental period (*experiment I*) (Fig. 1). The mean values $\pm$ SD for all 14 samples was, for volume, 0.45 ± 0.09 ml; for protein concentration, 9.04 ± 3.09 mg; and for amylase concentration, 9322 ± 2482 U/ml. The mean initial values for volume, protein concentration, and amylase concentration in *experiments II–VIII* did not differ from those in *experiment I*.

As shown in Fig. 2, intestinal bicarbonate infusion at 0.5 ml/h had no effect on bile-pancreatic secretion (*experiment II*). Likewise, intraduodenal infusion of pure bile (0.5 ml/h) during 3 h did not influence volume, protein output, or amylase output (*experiment III*, data not shown).

Infusion of 0.5 ml/h of bile-pancreatic juice was found to depress significantly amylase and protein output (*experiment IV*). Further, as seen in Fig.

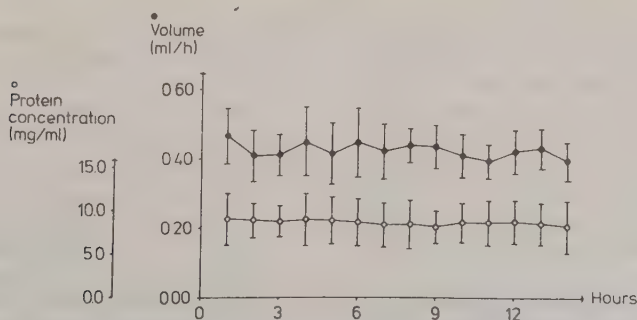


Fig. 1. Spontaneous secretory volume (●) and protein concentration (○) in conscious hamsters provided with bile-pancreatic fistulae (no. = 8). Mean $\pm$ SD.

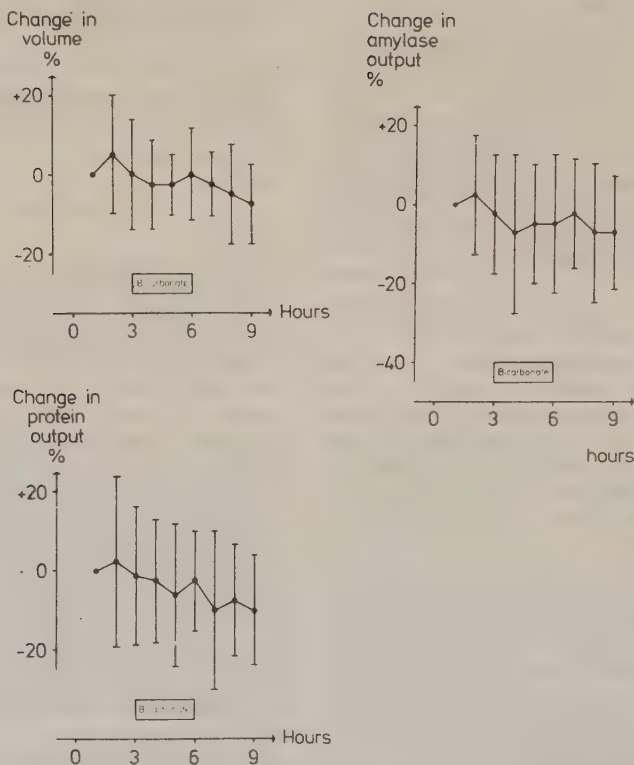


Fig. 2. Influence of intraduodenal bicarbonate infusion on volume, amylase output, and protein output in hamsters (no. = 3) provided with bile-pancreatic fistulae. Changes in percentage of initial values, all given as mean $\pm$ SD.

3, both amylase and protein output returned to the initial values a few hours after cessation of the infusion. The intestinal infusion of bile-pancreatic juice had only slight effects on the secretory volume (Fig. 3). When double the amount of bile-pancreatic juice (1.0 ml/h) was infused, the findings were similar except that the return of amylase and protein output to the initial values was somewhat slower (experiment V, data not shown).

As shown in Fig. 4, addition of trypsin inhibitor to the infused bile-pancreatic juice caused a marked increase in secretory volume and amylase and protein output (experiment VI). The effects

of the trypsin inhibitor compared with the changes seen after cessation of bile-pancreatic juice infusion are shown in Fig. 3.

Infusion of trypsin—like bile-pancreatic juice—into duodenum caused a marked depression of amylase and protein output (experiment VII). After interruption of the trypsin infusion a marked rise in both amylase and protein output was observed. Trypsin infusion did not affect secretory volume (Fig. 5). When the trypsin activity of the infusion solution was abolished by addition of trypsin inhibitor, there was a marked stimulation of both amylase and protein output and also of secretory volume (experiment VIII).

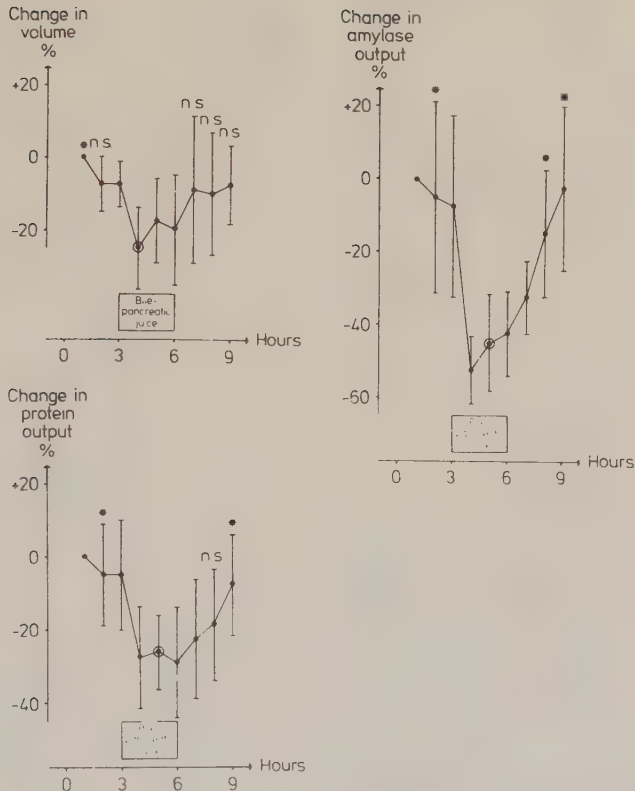


Fig. 3. Influence of reintroduction of bile-pancreatic juice on volume, amylase output, and protein output in hamsters (no. = 6) provided with bile-pancreatic fistulae. Changes in percentage of initial values, all given as mean $\pm$ SD. Significant differences from the reference point ($\odot$) are given; ns = not significant; * = $p < 0.05$.

Again, the effects of the trypsin inhibitor were similar to those seen after interruption of the trypsin infusion, as shown in Fig. 6.

Studies of long-term effects

Compared with the wet weight of the saline controls (0.29 ± 0.06 g, mean $\pm$ SD) the pancreatic weight of secretin-treated animals (0.33 ± 0.08) was not changed, whereas CCK (0.53 ± 0.08 , $p < 0.001$) and oral trypsin inhibi-

tor (0.43 ± 0.06 , $p < 0.05$) caused a significant enlargement of the gland. Ten days of repeated exogenous secretin injections did not influence pancreatic amylase or protein content, as shown in Fig. 7. On the other hand, CCK caused an increase of pancreatic amylase content to about 300% of the saline-treated group and of pancreatic protein content to about 175%. Oral trypsin inhibitor administration also caused a significant increase of both amylase and protein content, although to a lesser extent than CCK.

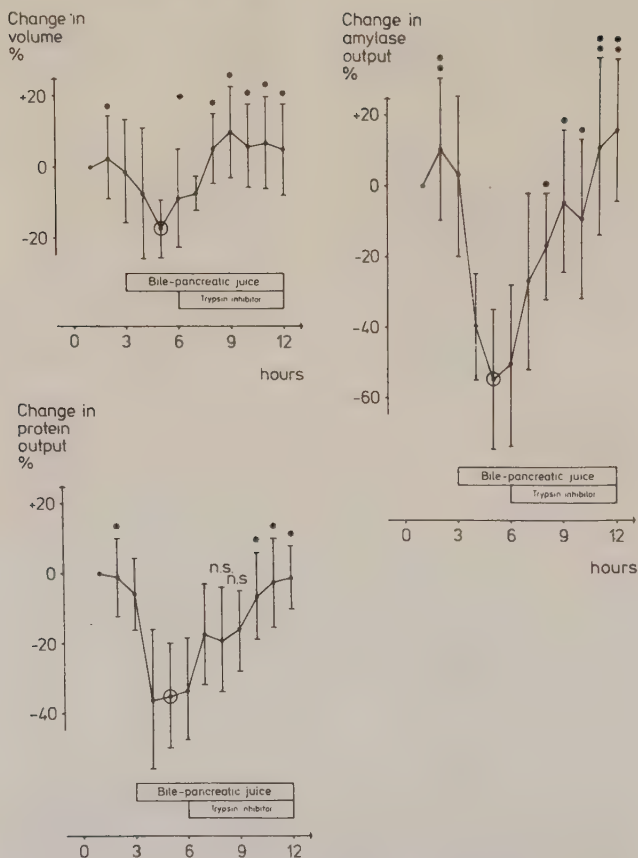


Fig. 4. Influence of reintroduction of bile-pancreatic juice with and without additional trypsin inhibitor on volume, amylase output, and protein output in hamsters (no. = 5) provided with bile-pancreatic fistulae. Changes in percentage of initial values, all given as mean $\pm$ SD. Significant differences from the reference point (●) are given. ns = not significant; * = $p < 0.05$; ** = $p < 0.01$.

DISCUSSION

The results of the present study show that the intraduodenal infusion of bicarbonate or bile—as in the rat (2)—does not influence pancreatic enzyme secretion in Syrian golden hamsters provided with bile-pancreatic fistulae. On the other hand, infusion of bile-pancreatic juice caused a

marked depression of amylase and protein output and a slight depression of secretory volume. When a trypsin inhibitor was infused together with bile-pancreatic juice, however, pancreatic secretion was restored to the initial values. Infusion of trypsin into the intestine also markedly suppressed the amylase and protein output but did

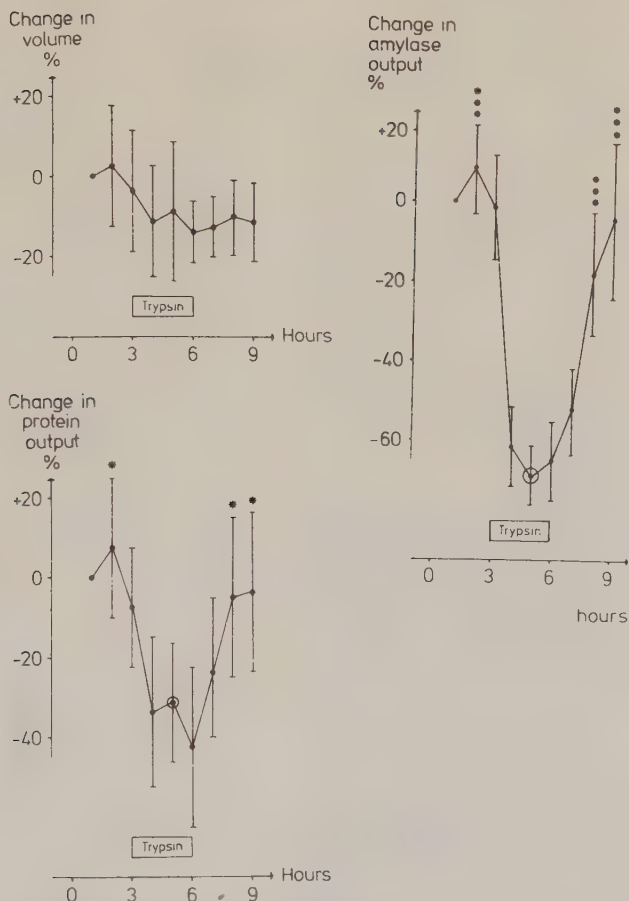


Fig. 5. Influence of intraduodenal trypsin infusion on volume, amylase output, and protein output in hamsters (no. = 6) provided with bile-pancreatic fistulae. Changes in percentage of initial values, all given as mean $\pm$ SD. Significant differences from reference point (○) are given. * = $p < 0.05$; *** = $p < 0.001$.

not influence the secretory volume. If trypsin inhibitor was added to the trypsin infusate, the suppression was abolished and pancreatic secretion restored to the initial value. These findings confirm that also in the hamster—as in the rat (2, 3) and pig (4)—exocrine pancreatic secretion is controlled by a negative feedback regulation exerted by intraluminal trypsin.

In the present study it was also found that repeated subcutaneous injections of cholecystokinin for 10 days caused a marked increase of pancreatic amylase and protein content. Similar, although less pronounced, changes were seen after oral administration of a trypsin inhibitor during a similar time period. Exogenous secretin administration had no effects on the variables

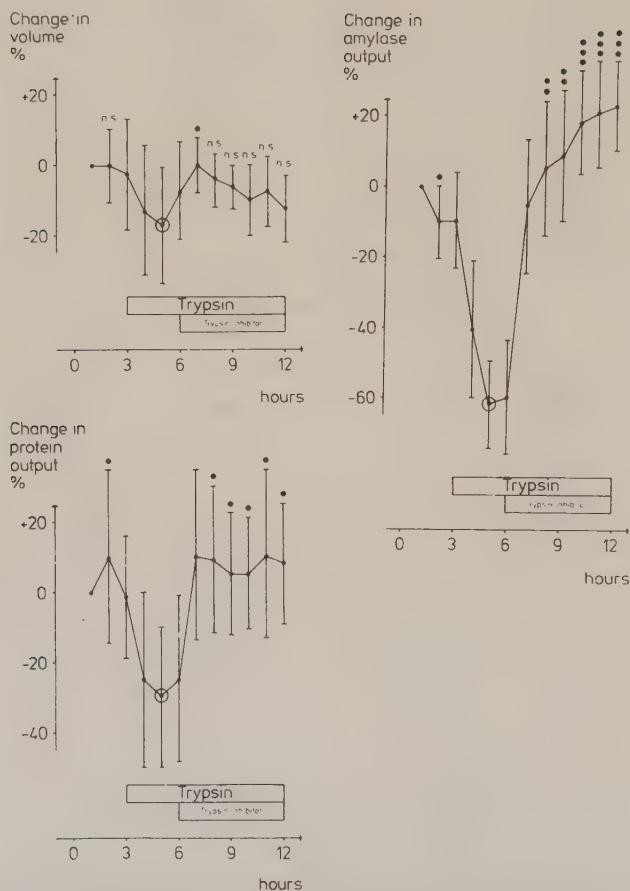


Fig. 6. Influence of intraduodenal trypsin infusion with and without additional trypsin inhibitor on volume, amylase output, and protein output in hamsters (no. = 6) provided with bile-pancreatic fistulae. Changes in percentage of initial values, all given as mean $\pm$ SD. Significant differences from reference point (○) are given. ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

studied. These results are consistent with the idea that cholecystikinin, like oral trypsin inhibitor, exerts trophic effects on the hamster pancreas and are in accordance with what has previously been shown in the rat (9).

In 1951 Annis & Hailenbeck (14) and Hong et al. (15) reported that in dogs provided with a pancreatic fistula, infusion of pancreatic juice into

the duodenum caused an inhibition of pancreatic secretion. On the other hand, Sale et al. (16) did not find evidence of a similar feedback regulation of exocrine pancreatic secretion in the dog. Magee & Hong (17) found that intraduodenal reinfusion of pancreatic juice to pigs caused a marked depression of pancreatic secretory volume and amylase secretion. Studies by Corring (18) and

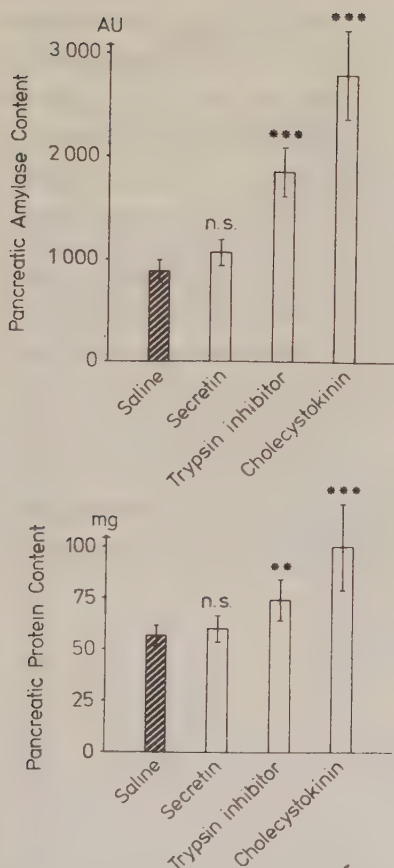


Fig. 7. Pancreatic amylase and protein content, respectively, in hamsters given repeated subcutaneous injections of saline (no. = 25), secretin (no. = 15), trypsin inhibitor (no. = 15), and cholecystokinin (no. = 15) for 10 days. Each column represents mean value $\pm$ SD. ns = not significant; ** = $p < 0.01$, *** = $p < 0.001$.

by Ihse & Lilja (4) suggest that the pig is subject to a feedback inhibition exerted by intestinal trypsin. Goldberg et al. (19) studied the recovery of pancreatic enzymes infused into the duodenum of human subjects with ileostomy and suggested, on the basis of their findings, that the pancreatic feedback inhibition from intestinal trypsin may exist in man. In a previous study of a single patient

we found more direct evidence that the proposed feedback mechanism also operates in man (5). Moreover, Osnes & Hanssen (7), using endoscopic cannulation of the pancreatic duct in man, found an inhibition of pancreatic secretion by duodenal infusion of pancreatic juice. On the other hand, Hotz et al. (6), using a triple-tube technique, did not find any evidence of a similar feedback mechanism in man. Thus, the definite solution of this problem must await further investigations.

It has been shown by McGuinness et al. that a diet composed of a raw soya flour—rich in trypsin inhibitors—induces acinar pancreatic cancer in rats (20) and sensitizes the rat pancreas to exogenous pancreatic carcinogens (21). It is also well established that in the rat long-term oral administration of trypsin inhibitor (8), like CCK (9), causes hypertrophy of the pancreas. Pancreatic regeneration induced by partial pancreatectomy (22) or ethionine (23) has also been reported to enhance the development of pancreatic cancer in rats given the carcinogen azaserine. However, for the rat there is no experimental model available which produces pancreatic cancer of ductal appearance. Since most human pancreatic cancers are ductal, the method recently described by Pour et al. (11) to induce ductal pancreatic cancer in the Syrian golden hamster by the use of nitrosamine derivatives is of utmost importance. The findings in the present paper that pancreatic secretion in the Syrian golden hamster is subject to a regulatory feedback mechanism exerted by intraluminal trypsin and that long-term oral trypsin inhibitor ingestion, like exogenous CCK administration, seems to have trophic effects also on the hamster pancreas may therefore provide directions for further studies of the role of exocrine pancreatic cell regeneration in the initiation and promotion of pancreatic carcinogenesis.

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STUDIES ON THE EFFECT OF CERULEIN ADMINISTRATION ON EXPERIMENTAL
PANCREATIC CARCINOGENESIS

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Running title: Cerulein and pancreatic carcinogenesis.

ABSTRACT

The influence of the cholecystokinin-analogue cerulein on induced pancreatic cancer in the Syrian golden hamster was investigated. Among hamsters given weekly subcutaneous injection of N-nitrosobis (2-hydroxypropyl)amine (BHP) in initial experiments 50 % succumbed within 30 weeks when a dose of 125 mg BHP per kg bw was used and within 25 weeks after the double dose. Therefore an induction time of at most 24 weeks was used in the subsequent experiments. Administration of cerulein (2 μ g twice daily for 5 days a week) for 18 or 22 weeks caused an increase of pancreatic wet weight by about 100 % and of pancreatic protein content by 73 % (18 weeks). BHP did not influence the pancreatic weight neither in hamsters given cerulein nor in those given saline injections. BHP (125 mg/kg bw) made 44 % of the animals tumor-bearing after 18 weeks and 73 % after 22 weeks. When BHP was given in a dose of 250 mg/kg bw 100 % of the animals had pancreatic tumors after 22 weeks. At neither dose and neither time interval did cerulein influence the number of tumor-bearing animals, number of cancer-bearing animals, number of tumors per tumor-bearing animal or cancers per cancer-bearing animal. No morphological differences were found within the lesions of animals given only BHP as compared to those given cerulein in addition. All lesions were of ductal appearance. Also, the distribution of tumors was similar irrespective of the treatment given. The results show that cerulein does not influence experimental pancreatic carcinogenesis in the Syrian golden hamster, possibly reflecting that cerulein and BHP primarily act on different target cells.

KEY WORDS: cerulein; pancreatic cancer; Syrian golden hamster.

In recent years it has come to be recognized that gastrointestinal hormones of the cholecystokinin (CCK) group exert trophic effects on the pancreas (1, 2). There are also some evidence that regenerating tissue may have increased susceptibility to carcinogens (3). This raises the question whether factors resulting in an excessive and sustained release of gastrointestinal hormones may influence the development of pancreatic cancer e.g. by sensitizing the gland to environmental carcinogens. On the other hand it has recently been suggested that CCK even may have inhibiting effects on induced pancreatic carcinogenesis in the Syrian golden hamster (4).

The purpose of this study was to investigate whether the CCK-analogue cerulein exerts any influences - stimulatory or inhibitory - on growth of induced pancreatic cancer in the Syrian golden hamster.

MATERIAL AND METHODS

Inbred 8 weeks old Syrian golden hamsters from own breeding weighing on an average 100 g at the start of the experiments were used. In all experiments equal representations of both sexes were included. During the experimental period the animals were housed in groups of two in plastic cages and kept on a standard pellet diet composed according to actual international specifications and containing the following raw material: fish protein concentrate, soya meal (roasted), fodder yeast, cereals, wheat sprouts, animal fat, soya oil, vitamine concentrate and minerals. The pellet diet was delivered by Anticimex AB, Stockholm, Sweden. The animals had free access to tap water.

N-nitrosobis(2-hydroxypropyl)amine (BHP) was purchased from Ash Stevens Co., Detroit^R, Michigan, and the cholecystokinin-analogue cerulein (Ceruletide^R) was kindly donated by Pharmitalia Research Laboratories, Milano, Italy.

In experiment I 90 hamsters were given subcutaneous injections for life of 125 (n = 60) or 250 (n = 30) mg/kg body weight of BHP in 0.9 % saline. This experiment was done in order to choose a suitable duration of the period of the exposition to the carcinogen with regard to survival. Therefore, the mortality rate in relation to time was the only parameter recorded.

In experiment II hamsters were given weekly subcutaneous injections of BHP (125 mg/kg body weight) for 18, 20, 22 or 24 weeks. In each group about half of the hamsters underwent a sham operation in ether anaesthesia via a mid-line incision comprising thorough palpation and inspection of all intraabdominal organs. The purpose of this experiment was primarily to study the influence of time on the frequency of BHP-induced pancreatic tumors, as different results in this respect are reported from different laboratories (5, 6).

Experiment III was performed to study the effects on the hamster

pancreas of cerulein per se and in combination with the carcinogen (BHP). Pancreatic weight and in some animals pancreatic protein was measured. The frequency, distribution and histological appearance of the induced pancreatic tumors were recorded. The duration of carcinogen exposition chosen in this experiment was based on the findings of experiments I and II and selected in order to allow evaluation of possible stimulation as well as inhibitory effects of cerulein.

Two groups of hamsters ($n = 12$ in each group) were given subcutaneous injections of 2 ug of cerulein in 0.9 % saline twice daily for five days a week during 18 or 22 weeks, respectively. Their respective control groups ($n = 16$ and 10, respectively) were given saline injections in the same amount (0.2 ml) and at the same time intervals. Two other groups of eleven hamsters were given cerulein as described above and in addition 125 mg/kg body weight of BHP once weekly for 18 or 22 weeks, respectively. The control groups (11 animals in each group) were given the same dose of BHP but saline injections instead of cerulein. Finally, one group of 12 animals was given cerulein as described above and in addition 250 mg/kg body weight of BHP once weekly whereas 12 controls were given the same dose of BHP but saline injections instead of cerulein.

At the end of the experiment the animals were killed by an overdose of ether and routine necropsies performed. The pancreas was rapidly removed, its wet weight determined, put in 10 % buffered formalin and embedded in paraffin. An average of 6-8 step sections were prepared from the gastric and the splenic lobes of the pancreas and 2-4 step sections from the duodenal lobe. Using this technique the distance between each section was at most 1.5 mm. Staining was performed with hematoxylin and eosin. The exact anatomical location of all histological lesions was recorded on a diagram of the pancreas. The diagnostic criteria used are in accordance with those described by Pour and Wilson (7).

The data obtained were subjected to statistical analysis by use of Fisher's exact test.

RESULTS

Experiment I

As can be seen in figure 1 half of the animals succumbed within 30 weeks after given a weekly dose of 125 mg/kg body weight of BHP and after 25 weeks when given the double dose. Based on these findings an induction period of at most 24 weeks was used.

Experiment II

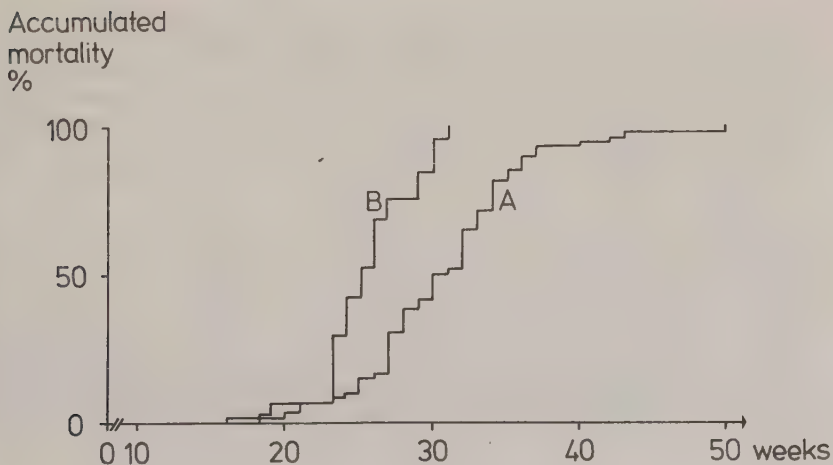
As shown in Table I the tumor frequency increased with the duration of exposition to the carcinogen. Among all tumor-bearing animals the proportion of hamsters with malignant tumors was 0.5, 0.7, 0.6, and 0.6 after 18, 20, 22 and 24 weeks of induction, respectively.

Table I.

Influence of time on frequency of BHP-induced tumors in hamsters.
BHP given as weekly subcutaneous injections (125 mg/kg bw) for
18, 20, 22 and 24 weeks.

	18 weeks	20 weeks	22 weeks	24 weeks
Number of animals	22	21	22	27
Tumor-bearing animals (%)	10 (45%)	14 (67%)	16 (73%)	25 (93%)
Cancer-bearing animals (%)	5 (23%)	10 (48%)	10 (45%)	18 (67%)

Fig 1. Accumulated mortality rate in hamsters given subcutaneous injections for life of 125 (A), or 250 (B) mg/kg body weight of BHP in 60 and 30 animals, respectively (experiment I).



Experiment III

Administration of cerulein for 18 or 22 weeks caused an increase of the wet weight of the pancreas by about 100 % (Table II). Also in hamsters given different doses of BHP (125 or 250 mg/kg body weight) cerulein had a similar effect on pancreatic weight. Neither doses of BHP caused any changes of pancreatic weight as compared to saline treated controls (Table II). In a few animals ($n = 6$) given cerulein for 18 weeks the pancreatic protein content was increased by 73 % as compared to saline treated controls ($n = 6$).

Table II. Influence of cerulein on pancreatic wet weight in hamsters given a weekly subcutaneous injection of saline, 125 mg/kg bw of BHP (BHP x 1) or 250 mg/kg bw of BHP (BHP x 2) for 18 or 22 weeks.
Mean $\pm$ SD

	Saline	Cerulein	BHP x 1	BHP x 1 Cerulein	BHP x 2	BHP x 2 Cerulein
18 weeks	0.36 $\pm$ 0.05 (n = 16) p < 0.001	0.75 $\pm$ 0.08 (n = 12)	0.46 $\pm$ 0.09 (n = 11) p < 0.01	0.85 $\pm$ 0.07 (n = 11)	-	-
22 weeks	0.42 $\pm$ 0.08 (n = 10) p < 0.01	0.75 $\pm$ 0.10 (n = 12)	0.37 $\pm$ 0.05 (n = 11) p < 0.02	0.84 $\pm$ 0.26 (n = 11)	0.42 $\pm$ 0.08 (n = 12) p < 0.05	0.74 $\pm$ 0.23 (n = 12)

In this study 125 mg/kg body weight of BHP given subcutaneously once a week for 18 weeks made 44 % of the animals tumor-bearing (Table III); after an induction period of 22 weeks the same dose of BHP caused tumors in 73 % of the animals. Hamsters given cerulein in addition had a similar tumor frequency. Also in hamsters given 250 mg/kg body weight of BHP for 22 weeks cerulein did not influence the tumor frequency. Further, the proportion of animals with malignant tumors was uninfluenced by cerulein administration.

As shown in Table IV the total number of tumors in the different groups was not significantly influenced by the addition of cerulein to the BHP-treatment, irrespective of dose of BHP or induction time. Further, the addition of cerulein did not significantly affect the number of tumors per animal or tumor per tumor-bearing animal (Table IV). All tumors (benign as well as malignant) were of ductal appearance. The benign tumors were classified as duct cystadenomas (42 %) or duct papillomas (36 %), but also papillary cystadenomas (19 %) or solitary adenomas (3 %) were seen. In saline treated control

Table III. Influence of cerulein on number of tumor-bearing and cancer-bearing animals. BHP x 1 = 125 mg/kg bw BHP weekly. BHP x 2 = 250 mg/kg bw weekly. In no respects any statistical differences were found.

	18 weeks		22 weeks			
	BHP x 1	BHP x 1 Cerulein	BHP x 1	BHP x 1 Cerulein	BHP x 2	BHP x 2 Cerulein
Number of tumor-bearing animals	5/11	8/11	8/11	8/11	12/12	10/12
Number of cancer-bearing animals	3/11	0/11	6/11	5/11	11/12	10/12

Table IV. Number of benign tumors, carcinomas in situ and adenocarcinomas in hamsters treated with BHP with and without addition of cerulein. In no respects were any statistical differences found between corresponding BHP and BHP-cerulein groups.
BHP x 1 = 125 mg/kg body weight weekly
BHP x 2 = 250 mg/kg body weight weekly

Type of tumor	18 weeks		22 weeks			
	BHP x 1 n = 11	BHP x 1 Cerulein n = 11	BHP x 1 n = 11	BHP x 1 Cerulein n = 11	BHP x 2 n = 12	BHP x 2 Cerulein n = 12
Benign tumors	11	9	24	24	39	37
Carcinoma in situ	0	0	2	3	1	0
Adenocarcinoma	5	0	9	17	18	30
Total number of tumors	16	9	35	44	58	67
Number of tumors per animal	1.5	0.8	3.2	4.0	4.8	5.6
Number of tumors per tumor-bearing animals	3.2	1.1	3.8	5.5	4.8	6.7

animals no tumors were found, while in those given cerulein but no carcinogen one hamster had a papillary serous adenoma and another hamster had multiple cystadenomas in addition to a solitary adenoma.

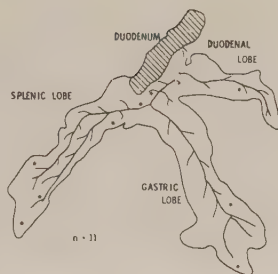
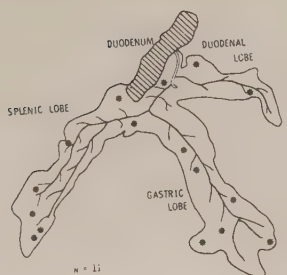
Marked dilatation or distension of the ducts and/or ductuli and hyperplasia of the ductal or ductular epithelium was a common finding in animals given BHP. Also in a few animals of the saline control group mild similar changes were noticed. Cerulein per se caused moderate ductal hyperplasia in about one fifth of the animals but the degree of dilatation and distension of the ducts or ductuli were similar to that of the control group. In animals given the carcinogen cerulein did not seem to influence the frequency of hyperplasia or duct cell dilatation or distension.

In figure 2 the distribution of tumors within the pancreas after the different types of treatment is shown. It is obvious that there was no preferential area for tumor development in the BHP-treated animals, irrespective if cerulein was given or not.

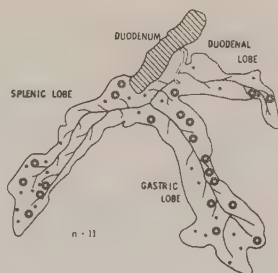
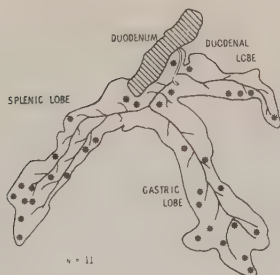
without cerulein

with cerulein

BHP
18 weeks



BHP
22 weeks



BHP x 2
22 weeks

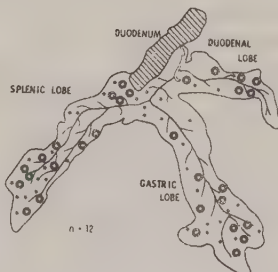
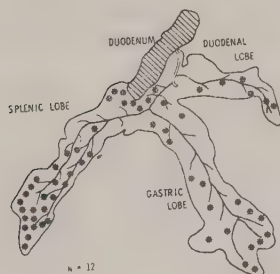


Fig 2. Distribution of tumors (benign and malignant) in hamsters given weekly injections of 125 mg/kg bw of BHP for 18, 22 weeks or 250 mg (BHP x 2)/kg bw of BHP for 22 weeks with or without the addition of cerulein injections (2 μ g) twice daily. Each dot represents one tumor.

DISCUSSION

The results of the present study show that half of the animals succumbed within 30 weeks if they were given a weekly subcutaneous injections of 125 mg/kg body weight of BHP and within 25 weeks after the double dose. It was also found that sham operation did not affect tumor frequency. Long-term subcutaneous administration of the CCK-analogue cerulein increased the wet weight and protein content of the pancreas suggesting that cerulein acts as a trophic factor on the pancreas of the hamster. Weekly administration of the carcinogen BHP in a dose of 125 mg/kg body weight for 18 or 22 weeks caused pancreatic tumors in 45 and 73 % of the animals, respectively. The addition of the trophic factor cerulein in adequate doses did not cause any significant - stimulatory or inhibitory - modulation of the development of pancreatic tumors.

One explanation of the failure of cerulein to affect pancreatic carcinogenesis in the present study may be that the acinar cells are known to be the main target cells for the action of this factor or hormone. Most carcinomas of the exocrine pancreas of the human like those induced experimentally in the hamster are composed of cells with the morphological appearance of ductal epithelium. As the cells generally contain mucin and are devoid of zymogen granule it is widely assumed that this type of pancreatic cancer arises from the ductal epithelium (8). Further, the incidence of carcinoma in situ and papillary hyperplasia of the ductal epithelium has been reported to be significantly more common in patients with pancreatic cancer than in matched autopsy control cases (9). Recent experimental data, however, seem to implicate the acinar cell as the main target for carcinogenic influences (10, 11, 12). Thus, it has been shown that the acinar cells can be stimulated to proliferate under appropriate conditions (13). During the early stages of carcinogenesis increased mitotic activity, measured by ³H-thymidine nuclear labelling in the pancreatic acinar cell, has been described in the hamster (14). A single injection of 4-HAQO (4-hydroxyaminoquinoline 1 oxide) (22.5 mg/kg body weight) caused marked necrosis of the exocrine acinar pancreas within 48 hours (15) and we have found acute, fatal hemorrhagic pancreatitis in the hamster following a single injection of 2 500 mg/kg body weight of BHP (unpublished observations). Histogenetic studies of induced pancreatic cancer in the guinea-pig led to the suggestion that the differentiation and retrodifferentiation of acinar cells may result in the formation of cells with apparent ductal or ductular phenotype (11). Different authors have found evidence in the hamster that ductal and ductular cells under the influence of different carcinogens may arise from acinar cells and play a role in the genesis of neoplastic cells (12, 16). The resolution of the controversy about the role of the ductal or acinar cells as the cells of origin of pancreatic cancer must await further investigations. If anything the results of the present study may favour the ductal cells in this respect. Whether secretin is capable to affect the nitrosamine induced pancreatic

cancer in the hamster remains to be elucidated.

Johnson et al (4) recently reported that CCK appears to protect the hamster from developing BHP-induced pancreatic carcinoma. These results, which are contrary to the findings of the present study, are somewhat difficult to interpret as CCK was given only once weekly, a dose which, e.g., is too low to cause any long-standing effect on the pancreas. Townsend et al (2) studied the effect of CCK and/or secretin on growth of pancreatic ductal cancers after inoculation of tumor cells into the cheek pouch of the hamster. They found that the combination of CCK and secretin increased tumor wet weight and DNA-content of the tumors, whereas cholecystokinin or secretin alone were without any effects in this respect. These observations are in accordance with the findings of the present study that the CCK-analogue cerulein alone did not promote pancreatic carcinogenesis in the Syrian golden hamster.

Longterm oral ingestion of trypsin inhibitors - like CCK and cerulein - causes hypertrophy of the pancreas in the rat (17). This probably reflects a feed-back stimulation of the pancreas exerted by intraluminal trypsin (18, 19) mediated via release of gastrointestinal hormones (17, 20). Levison et al (21) administered BHP to rats fed a raw soya flavour diet (rich in trypsin inhibitors) and found hyperplastic and adenomatous pancreatic noduli and the development of pancreatic adenocarcinoma. They concluded that the diet augmented the carcinogenicity of pancreatic carcinogens in the rat. Konishi and coworkers (22) reported enhanced pancreatic carcinogenesis in rats given a single dose of 4-HAQO three days after pancreatic resection. Their conclusion was that the regeneration caused by pancreatic resection was stimulatory to carcinogenesis, however, 4-HAQO differs from BHP in primarily affecting the acinar cells. Studies on the effects of oral administration of trypsin inhibitors on pancreatic carcinogenesis in the hamster are of great interest.

In summary, the results of the present study do not show any promotion of pancreatic carcinogenesis (BHP) by cerulein in the hamster although a marked increase of pancreatic wet weight and protein content was induced. Further studies on possible stimulatory effects by gastrointestinal hormones on experimental pancreatic cancer are, however, important e.g. by using exogenous secretin administration or oral trypsin inhibitor ingestion in combination with a carcinogen.

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STUDIES ON THE EFFECT OF CHOLECYSTOKININ, SECRETIN, AND
ORAL TRYPSIN INHIBITOR ADMINISTRATION ON EXPERIMENTAL
PANCREATIC CARCINOGENESIS

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ABSTRACT

The influence of cholecystokinin (CCK), sekretin, a combination of the two hormones, and the influence of oral trypsin inhibitor on early ductal pancreatic carcinogenesis was studied in the Syrian golden hamster.

Repeated injections of CCK alone or in combination with sekretin, and long-term oral administration of trypsin inhibitor for 6 or 9 weeks caused - contrary to repeated injections of sekretin alone - an enlargement of the pancreatic gland in Syrian golden hamsters given weekly subcutaneous injections of 10 mg N-nitrosobis(2-oxypropyl)amine/kg bw. No influence was, however, found in the total number of tumors and cancers, the number of tumors and cancers per animal, and the number of tumor- or cancer-bearing animals, respectively. Similarly the appearance of pre-neoplastic changes (tubular complexes or ductal proliferations with marked atypia) was unaffected by the treatment.

The results do not support the idea that regenerating pancreatic tissue is more susceptible to carcinogenic influences and question a role for dietary factors as modulators of the development of pancreatic cancer.

Key words: Cholecystokinin, N-nitrosobis(2-oxypropyl)amine, Pancreatic carcinogenesis, Sekretin, Syrian golden hamster, Trypsin Inhibitor.

It is well known that cholecystokinin (CCK) and gastrin exert trophic effects on the rat pancreas (20). Recently it has been shown that also CCK, and the octapeptide of CCK, are pancreatotrophic in the Syrian golden hamster (1,3,12). This is of interest for two reasons. Firstly, a useful experimental model for ductal pancreatic cancer is established in the hamster (13), and secondly, it has been suggested that trophic factors may sensitize the target organ to carcinogens (15). Thus, partial pancreatectomy - a known stimulus to growth of the remaining pancreatic tissue - has been shown to promote induced acinar pancreatic cancer in the rat (8) and exogenous gastrin administration enhanced tumor induction in an experimental model for gastric cancer (18). In a previous study, however, we did not find any stimulatory effects of the CCK-analogue cerulein on induced carcinogenesis in the Syrian golden hamster (1).

Secretin has not been shown to possess any convincing trophic effects on the pancreas (3,20). It is, however, well known that secretin and CCK each augments the primary pancreatic action of the other (17). Recent data suggest that secretin in the presence of a background activity of CCK has some stimulatory influence on pancreatic growth and DNA synthesis (1).

Long-term oral administration of trypsin inhibitors to rat and hamster causes enlargement of the pancreas and an increase of its content of protein and digestive enzymes (3,6). In both species trypsin within the intestinal lumen exerts a negative feed-back regulation of pancreatic secretion (3,7). This regulatory mechanism is mediated by humoral factor(s), probably of the CCK-type (16,19). It is also of interest that ingestion of a trypsin inhibitor diet caused spontaneous adenomas and a few adenocarcinomas in the rat (9) and enhanced the development of induced acinar pancreatic cancer (10).

In the present study we investigated the influence of CCK and secretin separately and in combination and the influence of oral trypsin inhibitor administration on early ductal pancreatic carcinogenesis in the Syrian golden hamster.

MATERIAL AND METHODS

Animals. Inbred 8 weeks old Syrian golden hamsters from own breeding weighing on an average 100 g at the start of the experiments were used. Equal representation of both sexes were included. During the experimental period the animals were kept in groups of two in plastic cages. If not otherwise stated they were fed a standard pellet diet and had free access to tap water.

Diets. The standard pellet diet used was composed according to actual international specifications and contained the

following raw material: fish protein concentrate, roasted soya meal, fodder yeast, cereals, wheat sprouts, animal fat, soya oil, vitamin concentrate and minerals (Anticimex AB, Stockholm, Sweden). The trypsin inhibitor diet was composed of raw soya bean flour supplemented with vitamins and minerals, Diasoy^R (Special Diet Services Ltd., Essex, England). Heated soya bean flour, Soyolk^R (Special Diets Services Ltd., Essex, England), was used as control diet - the flour steam heated under pressure (130°C for 66 min) followed by drying (80-40°C for 1 h), has been found to destroy the soya bean trypsin inhibitor (10).

Gastrointestinal hormones. Cholecystokinin was obtained from KabiVitrum, Stockholm, Sweden, as was secretin.

Carcinogen. N-nitrosobis(2-oxypropyl)amine (BOP) was obtained from Ash Stevens Inc., Detroit, Michigan, USA.

Experimental design. One hundred and eighty hamsters were used and divided into six groups of 30 animals. Four of the groups were given subcutaneous injections twice daily five days a week of 0.5 ml 0.9% saline, 2 Ivy Dog Units (IDU) of CCK, 2 Clinical Units (CU) of secretin or a combination of both hormone doses, respectively. The remaining two groups of animals were fed ad libitum the active and inactivated trypsin inhibitor diets, respectively. Each animal of the study had a weekly subcutaneous injection of 10 mg/kg bw of BOP in 0.9% saline. After six weeks 15 animals from each group were randomly selected and killed whereas the remaining half of the animals were killed after another three weeks.

After sacrifice the pancreas was rapidly removed, its wet weight determined, fixed in 10% buffered formalin and embedded in paraffin. An average of 6-8 step sections were prepared from the gastric and the splenic lobes of the pancreas and 2-4 step sections from the duodenal lobe. Using this technique the distance between each section was at most 1.5 mm. Staining was performed using hematoxylin-eosin. The exact anatomical location of all histological lesions was recorded on a diagram of the pancreas. The histological criteria used for diagnosis were in accordance with those of Pour et al (13).

Calculations. Statistical evaluations were done using Student's t-test.

RESULTS

There were no statistically significant differences in body weight neither between the 6 and 9 week-groups nor between the different treatment groups.

Administration of CCK or CCK combined with secretin, or ingestion of the trypsin inhibitor diet caused a significant

Table I Influence of repeated injections of CCK, secretin, and long-term oral administration of trypsin inhibitor, (TI) on body weight and pancreatic wet weight of Syrian golden hamsters given weekly subcutaneous injections of 10 mg BOP/kg bw. Mean $\pm$ SD. x/ indicates significant difference ($p < 0.05$) from corresponding control groups.

Treatment groups	body weight (g)		pancreatic wet weight (g)	
	6 weeks	9 weeks	6 weeks	9 weeks
	(n = 15)	(n = 15)	(n = 15)	(n = 15)
Saline (control)	145 $\pm$ 19	131 $\pm$ 18	0.41 $\pm$ 0.08	0.43 $\pm$ 0.10
Secretin	131 $\pm$ 16	127 $\pm$ 18	0.44 $\pm$ 0.07	0.47 $\pm$ 0.10
CCK	135 $\pm$ 13	143 $\pm$ 12	0.55 $\pm$ 0.10 ^x	0.60 $\pm$ 0.08 ^x
CCK + Secretin	130 $\pm$ 16	137 $\pm$ 15	0.53 $\pm$ 0.08 ^x	0.55 $\pm$ 0.09 ^x

Heated TI (control)	138 $\pm$ 16	136 $\pm$ 14	0.40 $\pm$ 0.07	0.41 $\pm$ 0.07
Raw TI	136 $\pm$ 15	141 $\pm$ 19	0.50 $\pm$ 0.07 ^x	0.50 $\pm$ 0.06 ^x

Table II Influence of repeated injections of CCK, secretin, and long-term oral administration of trypsin inhibitor (TI) on number of tumors and cancers, and on number of tumor- and cancer-bearing animals in Syrian golden hamsters given weekly subcutaneous injections of 10 mg BOP/kg bw.

Treatment groups	no of tumors/no of TBA ^{x/}		no of cancers/no of CBA ^{xx/}	
	6 weeks	9 weeks	6 weeks	9 weeks
Saline (control)	0/0	9/6	0/0	2/2
Secretin	2/2	6/4	1/1	0/0
CCK	1/1	10/5	0/0	2/1
CCK + Secretin	2/1	11/5	1/1	7/5

Heated TI (control)	2/2	4/3	1/1	2/2
Raw TI	1/1	6/2	1/1	0/0

x/ TBA = Tumor-bearing animals

xx/ CBA = Cancer-bearing animals

increase in the wet weight of pancreas after 6 and 9 weeks by comparison with the respective control groups, whereas secretin alone was without effect. There were no differences between the wet weight at 6 and 9 weeks respectively (Table I)

As shown in Table II the total number of tumors and cancers, and the number of tumor-bearing and of cancer-bearing animals increased from 6 to 9 weeks, but there were no differences between the different treatment groups. Neither were there any differences in number of tumors and cancers per tumor- and cancer-bearing animal between the groups. No tumor had a diameter more than 2 mm.

Also concerning pre-neoplastic changes - tubular complexes and ductal proliferations with marked atypias - there was a significant progress from 6 to 9 weeks, but again there was no difference between the different treatment groups (Table III).

Treatment groups	No of TC/No of animals with TC		No of foci of DPA/No of animals with foci of DPA	
	6 weeks	9 weeks	6 weeks	9 weeks
Saline (control)	0/0	2/2	0/0	2/1
Secretin	0/0	4/2	0/0	2/2
CCK	0/0	7/5	0/0	10/5
CCK + Secretin	2/2	4/2	1/1	6/3

Heated TI (control)	0/0	3/1	0/0	3/2
Raw TI	0/0	5/2	0/0	7/4

Table III Influence of repeated injections of CCK, secretin, and long-term oral administration of active trypsin inhibitor (TI) on formation of pre-neoplastic lesions: tubular complexes (TC) and ductal proliferations with marked atypias (DPA), in Syrian golden hamsters given weekly subcutaneous injections of 10 mg BOP / kg bw

DISCUSSION

In the present study enlargement of the pancreatic gland was caused by repeated injections of CCK and CCK in combination with secretin for 6 or 9 weeks. Secretin alone did not influence the pancreatic wet weight. Oral trypsin inhibitor ingestion increased the pancreatic wet weight although to a lesser extent than CCK. As previous studies clearly have shown a trophic effect on the pancreas by both CCK and trypsin inhibitors in the Syrian golden hamster (3) it seems reasonable to assume that the enlargement of the pancreatic gland in this experiment was a consequence of a trophic action. Nevertheless, the different treatments did not influence the development of pancreatic tumors - benign or malignant - or pre-malignant signs such as ductal proliferation or tubular complexes (4,5). Using a similar experimental model we previously have found that the parameters used in this study - number of tumor(cancer)-bearing animals, number of tumors (cancers) totally and per animal, and the degree of ductal hyperplasia - are reliable in discriminating changes in the rate of tumor development (2). The findings of the present study, which are in accordance with a previous extensive study on the influences of cerulein on nitrosamine-induced pancreatic tumors (1), question that positive trophic effects make the target tissue more susceptible to carcinogens. As the BOP-induced cancer is of ductal appearance it, however, can be argued that the acinar cells are the target cells of CCK and of the trypsin inhibitor action but some authors have recently suggested that although the appearance of the cancer is ductal the origin may very well be the acinar cell (14).

Secretin has no known trophic effects on the pancreas and had in the present study no influence on tumor development. Therefore the results - if anything - indirectly support the ductal cell as the cell of origin of nitrosamine-induced cancer in the hamster. Further, as human pancreatic cancers generally are ductal the results also seem to question a role of dietary factors as modulators of the development of pancreatic cancer in man.

Oral trypsin inhibitors have been found to cause adenomatous pancreatic cancer in the rat (9) and to promote induced acinar pancreatic cancer in this species (10). As the trypsin inhibitors probably act via release of CCK from the intestine (16,19) experiments similar to those of the present study would be of interest using a model for acinar pancreatic cancer. Such studies could further elucidate the possible relationships between trophic influences and pancreatic carcinogenesis. For this purpose methods comprising transplantable acinar tumors also would be highly valuable.

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STUDIES ON THE FITNESS OF THE SYRIAN GOLDEN HAMSTER
FOR INVESTIGATIONS OF ESTROGEN EFFECTS ON INDUCED
PANCREATIC CARCINOGENESIS

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Running title: Estrogens and induced pancreatic cancer

ABSTRACT

As estrogen receptors (ER) and estrogen binding protein (EBP) have been described in normal pancreatic tissue and in pancreatic carcinoma in man we investigated the fitness of the model for experimental pancreatic cancer in the Syrian golden hamster for investigations of estrogen effects on induced pancreatic carcinogenesis.

Estradiol was without influence on basal pancreatic protein or amylase secretion; nor did estradiol change pancreatic weight, protein or amylase content in a 10 day experiment.

Estradiol binding protein, EBP, focusing at pH 4.5, was found in high amounts (1600 - 3100 fmol/mg protein) in healthy pancreas from all investigated hamsters, and was also in ancillary experiments found in comparable amounts in normal pancreatic tissue from cow, sheep, pig, guinea-pig, rat and mouse. In contrast to in normal tissue EBP was not detected in any of the induced pancreatic cancers in the hamster. Estrogen receptors, ER, were found in low concentrations (2 - 5 fmol/mg protein) in normal pancreatic hamster tissue and in even smaller amounts (0.5 - 2 fmol/mg protein) in the induced pancreatic cancers. Long-term estrogen administration (7 months) did not change the pancreatic content of ER and EBP, or the histological appearance of the gland.

We conclude that this otherwise useful experimental model for ductal pancreatic cancer is unsuitable for studies on estrogen-linked tissue-specific treatment of pancreatic carcinoma and therefore further studies of estrogen influence must be based on patients with exocrine pancreatic cancer.

Key-words: Estrogen binding protein, estrogen receptor, experimental carcinogenesis, pancreatic cancer, Syrian golden hamster.

The presence of specific receptors in or on a cell suggests hormonal influences on the metabolism or function of the cell and may further define hormone dependency on the disease state. Estrogen receptors (ER) (Sandberg & Rosenthal, 1979) and estrogen binding protein (EBP) have been described in normal pancreatic tissue (Pousette et al, 1982) and ER in pancreatic carcinoma (Greenway et al, 1981) in man. Although the physiological and pathophysiological significance of these proteins remains to be elucidated their existence in the pancreas gives directions for experimental and clinical studies on the possible effects of estrogens, anti-estrogens and estrogen-linked drugs in the treatment of pancreatic carcinoma.

The aim of this study was to evaluate the fitness of the animal model for ductal pancreatic carcinoma in the Syrian golden hamster (Pour & Wilson, 1980) for studies on estrogen or estrogen-mediated effects on pancreatic cancer.

MATERIAL AND METHODS

Six to eight weeks old male hamsters from own breeding, weighing 110-130 g at the start of the experiments, were used.

Studies on pancreatic secretion (Experiment I)

Hamsters were operated on through an abdominal incision exposing the duodenum and the bile-pancreatic duct. A silastic catheter with an inner diameter of 0.58 mm and a length of 15 cm (Dow Corning 602-105) was introduced directly into the bile-pancreatic duct close to the duodenum and secured in place with a silk ligature. The tip of the catheter was placed no longer than 3 mm from the duodenal wall. At the end of the operation each animal was given a subcutaneous injection of 10 ml of a solution containing 1 part Ringer and 1 part 5.5% glucose solution. The animals were kept in restraining cages and were allowed about 3 hours of surgical recovery before the experiments were started. All experiments were performed on conscious animals. During the experimental period food and water were withheld from the animals. Animals in which surgical complications occurred were excluded from the study.

The bile-pancreatic secretion was studied in 1 hour samples collected during 8 hours. After three hours polyestradiol-phosphate, Estradurin^R, (AB Leo, Helsingborg, Sweden) 1 mg/kg body weight was injected intravenously in 0.5 ml saline in five hamsters. This dose is known to exert carcinogenic effects on the rat kidney when given as repeated monthly injections (Karr et al, 1982). Control animals (n = 5) were given saline injections. The volume of the bile-pancreatic juice was measured. Protein was assayed according to the

method described by Lowry et al (1951). Amylase was determined by the Phadebas test, a gift from Pharmacia, Uppsala, Sweden.

Studies on long-term effects on pancreatic weight, protein and amylase content (Experiment II)

Ten hamsters were given a single dose of 1 mg/kg body weight of polyestradiolphosphate intramuscularly. Forty-five other hamsters - in groups of 15 - were given 2 clinical units (CU) of secretin (KabiVitrum, Stockholm, Sweden), 2 Ivy dog units (IDU) of cholecystokinin (KabiVitrum, Stockholm, Sweden) or 360 µg of somatostatin (KabiVitrum, Stockholm, Sweden) subcutaneously twice daily for ten days. Control animals (n = 25) got two daily subcutaneous saline injections for the same time period. On the tenth day of the experiment all hamsters were sacrificed - those given secretin, cholecystokinin, somatostatin or saline 12 hours after the last injection. The pancreas was rapidly removed and freed of connective tissue and lymph nodes and weighed and homogenized in a Sorvall Omnimixer. Protein and amylase content were determined using the same methods as in Experiment I.

In four hamsters (two male, two female), given polyestradiolphosphate 1 mg/kg body weight intramuscularly every fourth week during seven months in order to induce renal carcinoma, we got the opportunity to perform histological studies of the pancreas. As controls ten untreated hamsters of the same age and sex (five male, five female) were used. On an average 8-10 step sections were prepared from the gastric and splenic lobes of the pancreas and two or three from the duodenal lobe. The histological preparations were stained with haematoxylin-erythrosin.

Studies on estradiol receptors (ER), estradiol-binding protein (EBP) and progesteron receptors (PgR) in induced pancreatic tumors (Experiment III)

Pancreatic cancer was induced by weekly subcutaneous injections of 10 mg N-nitrosobis(oxypropyl)amine (BOP) (Ash Stevens Inc., Detroit, USA) per kg body weight for 24 weeks. After sacrifice by an overdose of ether the pancreas was removed and kept at -70°C for 1-2 weeks. Cancers - 5-10 mm in diameter - from six hamsters were analyzed for cytoplasmatic estradiol and progesteron receptor content. Tumor tissue (25-50 mg) were homogenized with an all-glass Potter Elvehjem homogenizer in 20 - 50 volumes of TEMN-buffer (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM and sodium azide 1.0 mM, pH 7.4) to get a protein concentration of 1-3 mg/ml. ER was measured with isoelectric focusing on polyacrylamide gel and PgR with the dextran coated charcoal method and Scatchard analysis as described previously (Norgren et al, 1982) with only one slight modification. The binding of ³H-estradiol

was thus studied over wider pH-gradient, 3.5-10 instead of 5.0-7.5 as described before (Norgren et al, 1982). The reason for this was to study an estradiol binding protein (EBP) focusing at pH 4.5. As far as the ER and EBP measurements were concerned most samples were also analyzed with 100 times excess of unlabeled estradiol for testing the specificity of the radioactive peak as measured by isoelectric focusing. The concentration of ER, EBP and PgR are expressed as femto (f) mols specifically bound ^3H -estradiol or ^3H -R5020 (PgR) per mg cytosol protein. The concentration of cytosol protein was measured according to the method described by Lowry (1951). As a comparison liver tissue from the six hamsters as well as pancreatic and liver tissue from seven healthy control hamsters were investigated for ER, EBP and PgR as was also one sample of pancreatic tissue from cow, sheep, pig, guinea-pig, rat and mouse, respectively.

Pancreatic glands from four hamsters given monthly poly-estradiolphosphate injections for seven months (vide supra) were investigated for the presence of ER, EBP and PgR.

In all experiments equal representation of both sexes was used.

RESULTS

Experiment I

As shown in table I an intravenous injection of polyestradiolphosphate - like saline - was without influence on unstimulated pancreatic protein or amylase output.

Table I Influence of a single intravenous injection of 1 mg/kg bw of polyestradiolphosphate (PEP) on protein and amylase output in hamsters with bile-pancreatic fistula. Mean $\pm$ SD. Within parentheses the numerical values corresponding to 100% are given in mg (protein) and U (amylase), respectively.

Hour	Change in protein output (%)		Change in amylase output (%)	
	PEP (n = 5)	Saline (n = 5)	PEP (n = 5)	Saline (n = 5)
1	100 (4.1 $\pm$ 1.2)	100 (3.9 $\pm$ 1.3)	100 (4010 $\pm$ 1313)	100 (4194 $\pm$ 1117)
2	103 $\pm$ 13	92 $\pm$ 19	108 $\pm$ 22	98 $\pm$ 12
3	98 $\pm$ 14	96 $\pm$ 7	104 $\pm$ 25	82 $\pm$ 27
4	98 $\pm$ 17	103 $\pm$ 21	95 $\pm$ 20	99 $\pm$ 3
5	90 $\pm$ 20	93 $\pm$ 23	88 $\pm$ 17	84 $\pm$ 23
6	91 $\pm$ 21	90 $\pm$ 5	97 $\pm$ 22	82 $\pm$ 33
7	92 $\pm$ 16	92 $\pm$ 18	101 $\pm$ 17	88 $\pm$ 35
8	87 $\pm$ 23	96 $\pm$ 11	89 $\pm$ 30	83 $\pm$ 21

Experiment II

A single injection of polyestradiolphosphate did not change the pancreatic weight, protein or amylase content of hamsters sacrificed 10 days after the injection by comparison with saline controls. Similary 10 days of somatostatin or secretin injections did not affect the pancreas. On the other hand long-term cholecystokinin administration caused a significant increase in pancreatic weight as well as in pancreatic protein and amylase content (figure 1).

Seven monthly injections of polyestradiolphosphate did not change the histological appearance of the pancreas.

Experiment III

In each of six pancreatic specimens from healthy hamsters a small peak of radioactivity was found at an isoelectric point of pH 6.5 indicating the presence of estradiol receptor (ER) at low concentrations (2-5 fmol/mg protein). A hundred times excess of unlabeled estradiol did however only partly eliminate the radioactive peak and as a consequence the real ER-concentration should thus be about half the amount. ER was also found in four of six samples of pancreatic cancer from hamster, but in even smaller amounts (0.5-2 fmol/mg protein). The radioactive peak was completely eliminated in two samples but only partly eliminated in the other two samples by excess of unlabeled estradiol. ER was found in the same amounts in each of the samples from the other six species. ER was not found in liver tissue from any of the hamsters of the study.

Neither in normal nor in cancerous pancreatic tissue were progesteron receptors (PgR) detected. Similarly PgR were not found in liver tissue from hamsters or from the other species studied.

Estradiol binding protein (EBP), focusing at pH 4.5, was found in healthy pancreatic tissue from all investigated hamsters ($n = 7$) at a concentration of 2.700 fmol/mg protein (range 1.600 - 3.100) (figure 2). EBP was also found in a comparable amount in the pancreas of cow, sheep, pig, guniea-pig, rat and mouse. A 100 times excess of unlabeled estradiol almost completely removed the radioactive peak at pH 4.5. In contrast to in healthy pancreatic tissue EBP was not detected in any of the induced pancreatic cancers. Finally, liver tissue from healthy as well as from cancer-bearing animals was found to be devoid of EBP.

Finally the pancreatic content of ER and EBP was the same in hamsters given seven monthly estrogen injections as in pancreatic glands of control hamsters and also in these hamsters no pancreatic PgR was found.

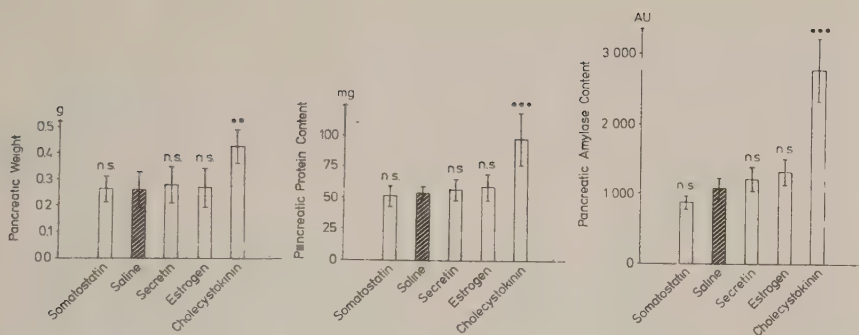


Fig. 1. Pancreatic weight, and pancreatic protein and amylase content, respectively, in hamsters given repeated subcutaneous injections of saline (no. = 25), secretin (no. = 15), somatostatin (no. = 15) and cholecystokinin (no. = 15) or a single intramuscular injection of polyestradiolphosphate (no. = 10). The duration of the experiment was 10 days. Each column represents mean value $\pm$ SD. ns = not significant, xx = $p < 0.01$, xxx = $p < 0.001$.

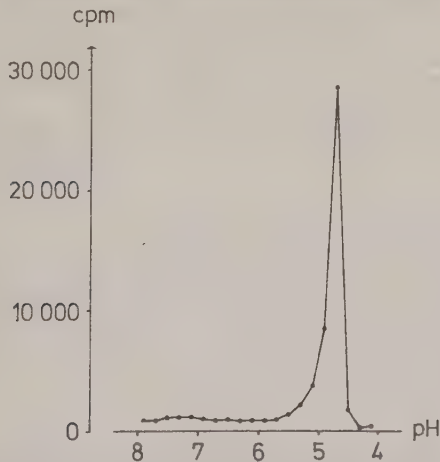


Fig. 2. Binding of ³H-estradiol to pancreatic tissue of normal Syrian golden hamster.

DISCUSSION

A model for induction of ductal pancreatic cancer has recently been described in the Syrian golden hamster (Pour, Krüger & Althoff, 1974). The presence of estrogen receptor proteins in the human pancreas - recently described (Sandberg & Rosenthal, 1970) - raises expectations that hormone manipulation might be of value in the treatment of pancreatic cancer. In this study we investigated the possible influence of estrogen on pancreatic function and growth and the presence of steroid binding receptor proteins in healthy and neoplastic hamster pancreas.

Sandberg & Rosenthal reported (1979) that polyestradiol-phosphate induces a profound disorganization of the microsomal structure of pancreatic cells in the dog. They also found a more irregular nuclear contour, fewer zymogens and empty ductal lumen. In accordance with these morphological data Boctor and Grossman (1977) observed significantly reduced amylase secretion within several hours after steroid administration. On the other hand estradiol - but not testosterone - restored zymogen granulation of acinar pancreatic cells to the initial levels in adrenalectomized male rats. In female rats reduction in zymogen granulation occurred only if adrenalectomy was combined with ovariectomy (Boctor & Grossman, 1977). Thus, these latter data sooner suggested a stimulatory role for estrogens in maintaining pancreatic function. In the present study - using a reliable method for direct studies of pancreatic secretion (Andrén-Sandberg & Ihse, 1983) - polyestradiolphosphate, however, in a dose known to induce renal carcinoma, had no apparent influence on exocrine pancreatic secretion in the hamster. Thus, it appears that exogenous administration of estrogens has no regulatory effects on the release of zymogen granules in this species.

Contrary to long-term administration of cholecystokinin, polyestradiolphosphate did not change the wet weight, protein or amylase content of the hamster pancreas. Similarly the histological appearance of the pancreas of hamsters given monthly injections of polyestradiolphosphate for seven months was the same as that of controls. Therefore, we conclude that long-term estrogen administration is without functional as well as morphological influences on the pancreas of the Syrian golden hamster.

Estrogen receptors have been found in rat pancreatic islets (Tesone et al, 1979), in normal exocrine pancreas of rat, guinea-pig and baboon (Sandberg & Rosenthal, 1979), in normal human pancreas (Sandberg & Rosenthal, 1979), in fetal human pancreas (Greenway et al, 1981) and in human pancreatic carcinoma (Stedman et al, 1980; Greenway et al, 1981). Satake et al (1982) found estrogen receptors in 44% of 16 DMBA-induced pancreatic adenocarcinomas in the Syrian golden

hamster investigated by Molteni et al (1979) were, however, negative with respect to estrogen receptors. As they chose a value of 6 fmol/mg protein as a dividing line of negative to positive binding, our findings of very low concentrations of ER in normal pancreas and even lower concentrations in pancreatic cancer support and extend the report by Molteni et al (1979). ER have been shown by autoradiography to be located preferably to the acinar cells (Sandberg & Rosenthal, 1979). As, however, also in normal pancreatic hamster tissue only low amounts of ER were found the lack of receptors in the tumors does not seem to reflect a ductal appearance of the cancers. Thus, unfortunately, the hamster model for ductal pancreatic cancer is not suitable for studies of therapeutic modalities related to the presence of ER in tumor tissue. It was therefore of interest when we found high amounts of EBP in normal hamster pancreas. Similar amounts of EBP were found in the pancreas of cow, sheep, pig, guinea-pig, rat and mouse and the presence of ERP in human pancreas has earlier been proved by Pousette et al (1982). As, however, EBP was not detected at all in the nitrosamine-induced cancers we must conclude that this otherwise useful experimental cancer model is unsuitable for studies on estrogen-linked tissue-specific treatment of the pancreatic carcinoma. The differences in content of ER and EBP between human ductal carcinoma and the induced ductal pancreatic carcinoma in the Syrian golden hamster seem to imply that future studies of possible estrogen influences on pancreatic cancer - in the absence of a useful experimental model - must be performed in patients with pancreatic cancer. Recently, however, we have detected an estramustine-binding protein, qualitatively different from the earlier described estrogen-binding protein, in the hamster pancreas (unpublished) giving directions for experimental studies of estramustine phosphate (Estracyt^R, a drug with known beneficial effects on prostatic carcinoma) in pancreatic cancer.

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UPTAKE AND BINDING OF ESTRAMUSTINE IN NITROSAMINE-INDUCED
EXOCRINE PANCREATIC CANCER IN THE SYRIAN GOLDEN HAMSTER

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Running title: Estramustine in experimental pancreatic cancer

SUMMARY

The uptake of estramustine, a nornitrogen mustard derivative of estradiol, in normal and cancerous pancreatic tissue was studied after intravenous administration of radiolabelled compound to normal and tumor-bearing Syrian golden hamsters. The binding of estramustine to cytosol fractions of the pancreatic and tumor tissue was studied in vitro by sucrose density gradient centrifugation.

The concentration of radioactivity was several times higher in the pancreas than in muscle and plasma. The radioactivity was also taken up in the tumors, although the concentration was lower than that of noncancerous pancreas.

Estramustine was only weakly bound to the estrogen-binding protein found in pancreas. This protein was not present in cancer tissue. Thus, it is not likely that the estrogen-binding protein is responsible for the pancreatic uptake of estramustine. Instead the binding studies revealed that estramustine uptake may be attributed to the binding to cytosolic proteins different from the estrogen-binding protein. The estramustine binding protein was present in pancreas as well as tumor tissue.

The results suggest that estramustine has properties which motivate further investigations on the possible use of the compound in patients with pancreatic cancer.

KEY WORDS

Estrogen binding protein, estramustine, Syrian golden hamster, pancreatic cancer.

The probability of curing a patient with pancreatic carcinoma is extremely low. Even at centers with special interest in this disease the over all 5-year survival is far below 1% (Brooks et al., 1981; Ihse et al., 1977; Gudjunsson et al., 1978). Radio-therapy has not substantially contributed to improvement of the prognosis (Dobelbower, 1981), nor has chemotherapy (Zimmerman, 1981).

Hormonal and hormone-linked treatment have been reported to be of value in malignant disease of the prostate (Jönsson, 1971; Edsmyr et al., 1982) and the breast (Manni, 1982). It was therefore of great interest when the presence of estrogen-receptors (ERs) and an estrogen-binding protein (EBP) were demonstrated in human pancreatic tissue (Sandberg & Rosenthal, 1979; Pousette et al., 1982) and ER in human pancreatic cancer (Greenway et al., 1981). These observations indicated new directions for studies with estrogens, anti-estrogenic agents and estrogen-linked cytotoxic drugs.

Among the estrogen-linked cytotoxic drugs estramustine phosphate (EMP, Estracyt^R) has been the most widely used (Edsmyr et al., 1982). EMP is a nornitrogen mustard derivative of estradiol-17 β -phosphate, which is rapidly dephosphorylated to estramustine (Figure 1) in the body (Gunnarsson et al., 1981). Estramustine may be considered an active metabolite because the compound has a direct cytotoxic activity in a human prostatic cancer cell line (DU 145) (Hartley-Asp & Gunnarsson, 1982). Estramustine is accumulated in the prostate via binding to a specific cytosolic protein (Forsgren et al., 1979; Björk et al., 1982).

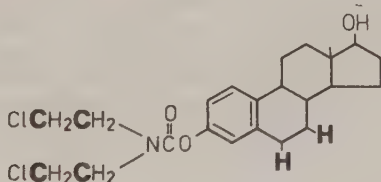


Fig. 1. Structural formula of estramustine. The positions of ^3H and ^{14}C -labelled atoms are given in extra bold types.

The aim of the present study, which is part of a series of experiments on estramustine phosphate in pancreatic cancer, was to evaluate the uptake and binding of estramustine in experimentally induced exocrine pancreatic cancer in the Syrian golden hamster.

MATERIAL AND METHODS

Animals and Chemicals

Eight weeks old, female Syrian golden hamsters from own breeding, weighing about 100 g at the start of the experiments, were used. Exocrine pancreatic cancer was induced by N-nitrosobis(2-oxypropyl)amine BOP, purchased from Ash Stevens Inc., Detroit, USA. The compound was administered subcutaneously, 10 mg BOP per kg bw, once weekly for 20 weeks. This dose schedule was chosen as it is known to cause malignant tumors in 100% of the animals (Pour & Wilson, 1980).

^3H , ^{14}C -estramustine (Figure 1) and ^3H -estramustine, were synthesized at AB Leo, Helsingborg, Sweden. The radiochemical purity, determined by thin layer chromatography, was not less than 95%. The specific activity was 4.8 mCi/mmol (^3H) and 2.1 mCi/mmol (^{14}C) for the double-labelled and 102 Ci/mmol for the single-labelled compound. Instagel, Carbosorb and Permaflour were bought from Packard Instrument Inc., Downers Grove, Illinois, USA. ^3H -Estradiol (55 Ci/mmol) and Aquassure were obtained from New England Nuclear Chemicals GmbH, Dreieichenhain, BRD.

Distribution studies

Fifteen hamsters treated with BOP as described above and 15 untreated hamsters of the same age were randomly allocated into groups of three. The animals were injected intravenously with 0.1 ml of ^3H , ^{14}C -estramustine, 5 mg/kg bw, prepared in polyethylene glycol 400, in the left femoral vein accessed under light ether anaesthesia.

At fixed times after dosing, 15 min, 1, 4, 8 and 24 h, the groups of animals were killed by exsanguination via the aorta under light ether anaesthesia. Immediately the pancreas, possible pancreatic tumors, liver, kidneys, and right hamstring muscles were removed, weighed and kept at -20°C until analyzed. Blood samples were cooled and sedimented at 2400 g for 10 min at 4°C and the ensuing plasma fraction was stored as the other specimens at -20°C until analyzed.

Samples (100-300 mg) of the various frozen tissues were combusted in a Packard Tri-Carb Sample Oxidizer, model 306. The tritiated water formed was determined in 10 ml of Aquassure; the $^{14}\text{CO}_2$ formed was trapped in 10 ml of Carbosorb and then mixed with 10 ml of Permaflour. The samples were counted in a Packard Tri-Carb Liquid Spectrometer, model 2650, or in a Philips PW 4510-00 Liquid Scintillator Analyzer for at least 10 min or 8×10^5 counts. Correction for quenching were made by the external channels ratio method. Tissues were analyzed at least in duplicates. The recovery of the combustion procedure was 102% (98-109) for ^3H and 101% (95-105) for ^{14}C . Plasma samples were determined directly in 5 ml of Aquassure or Instagel.

The area under the tissue concentration curve, AUC, of radioactivity after a single intravenous dose of ^3H , ^{14}C -estramustine was calculated for each organ and isotope by using the trapezoidal rule. The mean values of the concentrations of radioactivity in the different groups were used for the calculations.

In vitro binding studies

Pancreatic tissue was removed from five untreated Syrian golden hamsters and from five hamsters treated with BOP as described above. In the BOP-treated group the tumor tissue was separated from the tumor-free part of the pancreas; the latter was macroscopically normal but has in earlier experiments (Andrén-Sandberg et al., 1983) been found to have microscopically detectable precancerous lesions.

The specimens from each group were pooled and minced in three volumes of

GTEKD buffer (50 mM Tris-HCl, pH 7.4 at 25°C, containing 1 mM EDTA, 10 mM KCl, 0.25 mM dithiothreitol, and 10% v/v glycerol), and homogenized for five 5 s-periods (with 30 s cooling intervals) using an Ultra Turrax homogenizer (Janke and Kunkel, K.G., Straufen im Breisgau, BRD). The homogenates were centrifuged for 60 min at 105.000 g in a Beckman L5-65 Ultra centrifuge (SW 56 rotor) (Beckman Instruments Inc., Palo Alto, California, USA). The supernatants (cytosol fraction) were freed from the floating lipid layer and stored at -70°C until used.

The cytosols were thawed in an ice-bath and diluted to a final protein concentration of 2.4 mg/ml. Aliquots were labelled for 30 min at 30°C with 10-20 nM ^3H -estradiol or ^3H -estramustine in the absence or in the presence of a thousand-fold excess of unlabelled compound. The protein-bound radioactivity was obtained by gel filtration through small Sephadex G-25 Medium columns (Pharmacia Fine Chemicals, Uppsala, Sweden).

Aliquots of the protein-bound radioactivity, 0.2 ml, were loaded on to linear 5-20% w/w sucrose gradients, prepared in GTEKD buffer, and centrifuged for 23 h at 250,000 g as described elsewhere (Björk et al., 1982). After centrifugation the gradients were analyzed for absorbance at 280 nm and fractions of about 80 μl were collected and counted for radioactivity in 3 ml of Aquassure or Instagel as described above. Bovine serum albumin (BSA; 4.3 S) was run in parallel as a reference.

To study the stability of estramustine under the in vitro conditions used above, pancreatic cytosols (one ml) were incubated with ^3H -estramustine and extracted with ethyl acetate. The extracts were analyzed for estramustine and its degradation products by thin layer chromatography.

Protein was assayed according to Lowry et al. (1951).

RESULTS

Distribution studies

Distribution kinetics of radioactivity were studied in the normal, untreated hamsters after administration of ^3H , ^{14}C -estramustine. Figure 2 shows that the concentration of both isotopes was higher in the pancreas than in plasma and muscle throughout the 24 h interval. Fifteen minutes after the injection of ^3H , ^{14}C -estramustine, the concentration of ^{14}C in pancreas was about the same as that of ^3H , which indicates that parent estramustine is retained in this organ. Later, however, the concentration of ^{14}C was higher than that of ^3H , indicating that estramustine at least partly is hydrolyzed in the Syrian golden hamster.

The AUC-values were calculated for the 24 h period to get a quantitative comparison of the amounts of radioactivity distributed to the different tissues (Table I). The concentration of both isotopes was highest in the liver followed by the pancreas and kidneys.

When ^3H , ^{14}C -estramustine was given to BOP-treated animals, the concentrations of radioactivity in the different organs were about the same as in

Table I: Area under tissue concentration curve (% dose x h/g wet weight) of ^3H and ^{14}C after a single intravenous dose of ^3H , ^{14}C -estramustine (5 mg/kg bw) to Syrian golden hamsters. Mean $\pm$ SEM; n = 3.

Tissue	^{14}C	^3H
Plasma	2.47 $\pm$ 0.11	2.32 $\pm$ 0.10
Muscle	1.75 $\pm$ 0.12	1.11 $\pm$ 0.08
Liver	20.4 $\pm$ 1.6	10.4 $\pm$ 0.77
Kidneys	9.16 $\pm$ 0.60	4.44 $\pm$ 0.26
Pancreas	9.36 $\pm$ 0.63	5.10 $\pm$ 0.35

Table II: Area under tissue concentration curve (% dose x h/g wet weight) of ^3H and ^{14}C after a single intravenous dose of ^3H , ^{14}C -estramustine (5 mg/kg bw) to tumor-bearing Syrian golden hamsters. Mean $\pm$ SEM; n = 3.

Tissue	^{14}C	^3H
Plasma	2.68 $\pm$ 0.18	3.00 $\pm$ 0.25
Muscle	1.79 $\pm$ 0.09	1.28 $\pm$ 0.08
Liver	18.4 $\pm$ 2.0	8.53 $\pm$ 0.83
Kidneys	5.57 $\pm$ 0.46	3.06 $\pm$ 0.34
Noncancerous pancreas	6.95 $\pm$ 0.74	5.41 $\pm$ 0.60
Cancer	4.26 $\pm$ 0.22	2.51 $\pm$ 0.12

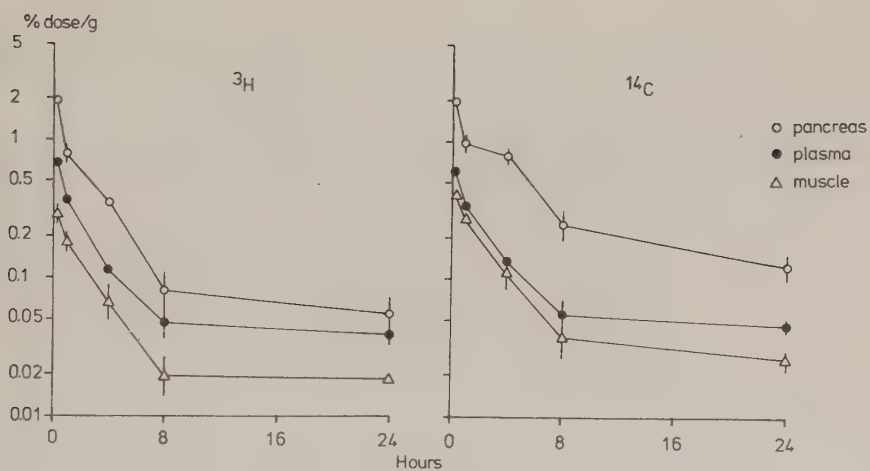


Fig. 2. Concentrations of ^3H and ^{14}C (mean $\pm$ S.E., $n = 3$) after a single i.v. dose (5 mg/kg) of $^3\text{H},^{14}\text{C}$ -estramustine to hamsters.

the corresponding organs in the experiments with untreated animals (Table II). This observation indicates that the distribution of estramustine in the Syrian golden hamster is uninfluenced by the carcinogen administration per se. In the tumor tissue the concentrations of ^3H and ^{14}C -activity were lower than in the noncancerous pancreas of the same animals. However, the concentrations of ^3H and ^{14}C in the tumor were two to three times higher than in the muscle, which suggests uptake of radioactivity also in the cancerous pancreas.

Protein binding

In the pancreas cytosol prepared from untreated animals, ^3H -estradiol showed more than ten times higher degree of binding than ^3H -estramustine (Table III). Furthermore, a thousand-fold excess of unlabelled estradiol gave almost a complete inhibition of ^3H -estradiol protein-bound radioactivity, while the same concentration of estramustine only displaced the radioactive compound to one third of the control sample. With ^3H -estramustine no inhibition was obtained with either an excess of unlabelled estramustine or estradiol.

Table III: Binding of ^3H -estradiol (E_2) and ^3H -estramustine (EM) to cytosolic proteins from pancreatic tissue of untreated female Syrian golden hamsters (I), and from macroscopically normal pancreas (II) and cancerous tissue (III) of BOP-treated animals (fmole/mg protein).

Tissue	^3H ligand	Control sample	+ a 1000-fold excess of E_2	+ a 1000-fold excess of EM
I.	E_2	3951	122	2667
	EM	350	390	426
II.	E_2	331	100	171
	EM	777	862	939
III.	E_2	100	100	100
	EM	375	452	397

In contrast to the normal pancreas, tumor tissue and noncancerous pancreas of the BOP-treated animals showed higher binding of ^3H -estramustine than ^3H -estradiol (Table III). Estradiol and estramustine radioactivity could not be displaced with a thousand-fold excess of estradiol or estra-

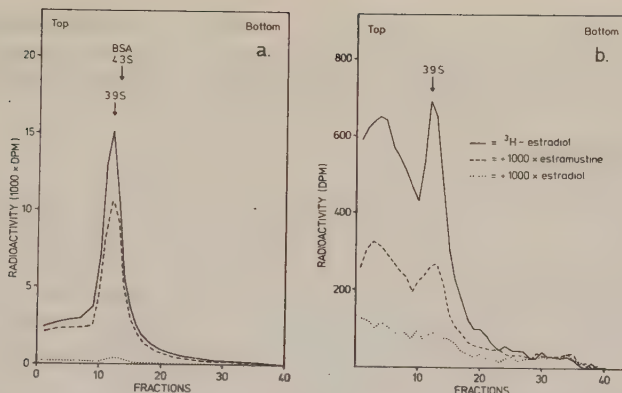


Fig. 3. Sedimentation profiles of ^3H -estradiol labelled cytosols prepared from pancreas of untreated animals (a) or from noncancerous pancreas of BOP-treated animals (b). Samples were labelled in the absence of or in the presence of a 1000-fold excess of unlabelled estradiol and estramustine respectively. Arrow indicates the position of bovine serum albumin; BSA, 4.3 S.

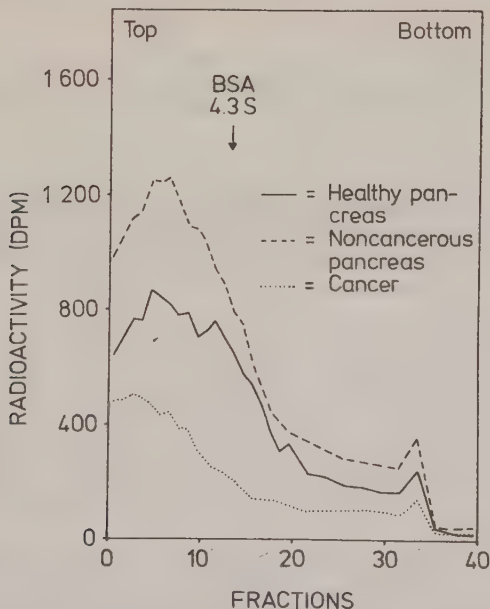


Fig. 4. Sedimentation profiles of ^3H -estramustine labelled cytosols prepared from pancreas of untreated animals and from noncancerous or tumor pancreatic tissue of BOP-treated animals. Arrow indicates the position of bovine serum albumin; BSA, 4.3 S.

mustine in the pancreatic tumor tissue. In the noncancerous pancreas, ^3H -estramustine was not inhibited with unlabelled estramustine or estradiol, while ^3H -estradiol could be displaced to more than 80% with estradiol and to almost 50% with estramustine.

Sedimentation analysis

The sedimentation profiles after sucrose density gradient centrifugation of the pancreatic cytosols are shown in Figures 3 and 4. In normal pancreas, protein-bound ^3H -estradiol sedimented as a 3.9 S complex, which was displaced almost completely with an excess of unlabelled estradiol and to 22% with estramustine (Figure 3 a). In the noncancerous pancreas of the BOP-treated animals a considerably lower radioactive peak was obtained, however, with the main radioactive peak sedimenting at the same position as for the healthy pancreatic cytosol; at 3.9 S (Figure 3 b). Unlabelled estradiol inhibited the protein-bound radioactivity to more than 80% and with estramustine to 54%. No distinct peak whatsoever was seen with the cytosol prepared from the tumor tissue.

The sedimentation curves obtained with cytosols labelled with ^3H -estramustine are shown in Figure 4. No distinct binding of ^3H -estramustine at 3.9 S was seen, but a broad peak sedimenting at 2-3 S. Moreover, protein-bound ^3H -estramustine was not displaced by an excess of unlabelled estramustine or estradiol, indicating non-saturable binding sites under the experimental conditions used.

No metabolism of estramustine was found under the in vitro conditions.

DISCUSSION

The distribution studies presented in this paper reveal an uptake of estramustine in the pancreas of normal Syrian golden hamster. The results also show that the compound is taken up in pancreatic tumors of BOP-treated animals even though the concentration in the tumors was lower than in the noncancerous pancreas of the same animals.

Previously EBP has been demonstrated in pancreatic cytosol of the Syrian golden hamsters (Andrén-Sandberg et al., 1982). The present results confirm the presence of EBP in the pancreatic cytosol from normal hamsters and demonstrate EBP also in the macroscopically normal pancreas of the BOP-treated animals. However, the degree of binding in this tissue was about ten times lower than that of normal animals. Furthermore, no significant estradiol binding could be detected in tumor cytosol of the BOP-treated hamsters, indicating that their pancreatic tumor lacks EBP.

As estramustine is a nornitrogen mustard derivative of estradiol we investigated if the binding of estramustine to EBP could be responsible for uptake of the compound. However, competition studies with labelled estradiol and unlabelled estramustine showed that estramustine was only very weakly bound to EBP. This finding together with the fact that EBP was not present in the pancreatic tumors strongly indicates that the uptake of estramustine in cancerous and noncancerous hamster pancreas was not caused by

binding of estramustine to EBP.

Estramustine is readily metabolized to estradiol in the rat, dog and man (Kirdani et al., 1977; Gunnarsson et al., 1981) and probably also in hamsters. Therefore it may be proposed that estradiol released upon hydrolysis of estramustine contributes to the pancreatic uptake of ^3H activity after administration of ^3H , ^{14}C -estramustine to hamsters. This hypothesis was, however, unvalidated by the finding that ^3H was taken up in pancreatic tumors in spite of the absence of EBP in this tissue and that more ^{14}C than ^3H was found in the tissue.

Instead the pancreatic uptake of estramustine appears to be attributed to the binding of the compound to a cytosolic protein which is different from EBP. Such a protein was demonstrated in the present study by incubation of ^3H -estramustine with cytosol preparations. In contrast to EBP, the estramustine binding protein was detected in cancerous as well as non-cancerous pancreas of the hamsters. Thus, the present results suggest that estramustine would be advantageous to compounds which bind to EBP, such as estrogens or antiestrogens, in the treatment of pancreas cancer. On the other hand EBP may be of value as a biological marker as there are qualitative and quantitative differences between cancerous and non-cancerous tissues. However, to ascertain if the results of the present hamster studies are relevant for human pancreatic cancer further studies are needed.

In human prostatic tumors, estramustine is bound to a cytosolic protein, which indicates a mechanism for selective accumulation of the active metabolite in the tumors of patients treated with estramustine phosphate (Fritjofsson et al. 1983). Ongoing studies with human pancreatic tissue have shown EBP to be present only in one out of five tumor specimens, while binding of estramustine has been detected in all of the tumors examined. These findings together with the results of the present hamster study may be extrapolated to humans indicating a similar mechanism for uptake of a cytotoxic compound, estramustine, in human pancreatic tumors.

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